DBP #1: Intracellular Core Processes in Biological Systems

PIs: Elowitz, Jensen, Phillips, Smolke

Overview of DBP #1

The first driving biological challenge area supported by the CBSA will be to examine the regulatory architectures that control the dynamics and stochastic behaviors of intracellular core processes. Projects supported under this DBP will examine the control systems governing core processes in micoorganisms including metabolism, polymerization, assembly, and signal transduction. The overall goal of this effort will be to develop a complete and predictive understanding of these control systems and to abstract the general architectures used by cells to control the dynamic behavior of these processes and how this relates to system function. Although within this DBP experiments will be conducted in single-celled organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, because the projects are examining core processes it is expected that these processes and likely their architectures will be conserved across organisms. Specifically, it is expected that the architectural principles developed in the projects supported under this DBP will scale with increasingly complex organisms.

The CBSA will initially support three projects under this DBP. The projects cover a diverse group of core processes and emphasize the elucidation of the control systems governing the dynamic and stochastic response of different conserved processes in cells. The first project explores how the cell uses structure and spatial organization as a control system and in particular examines its effects on the core processes of gene expression, polymerization and assembly, and protein activity. The second project explores how the cell interfaces dynamic control systems (acting at the level of gene expression and protein activity) with control systems based on spatial organization to control the dynamics of (or flux through) metabolic networks. The third project supported under this DBP explores the architecture associated with transcriptional control systems and their role in regulating the dynamics of cell behavior and population heterogeneity. Projects under this DBP utilize both the probing of endogenous systems (systems biology) and the construction and analysis of synthetic networks and engineered systems (synthetic biology) in order to examine relationships between regulatory architectures and core biological processes.

Christina Smolke 11/15/06 1:24 AM

Comment: Everyone please provide edits here. This is my first stab at an over arching summary for this DBP. Comments are appreciated.

Christina Smolke 11/15/06 1:17 AM

Comment: Maybe more specific core process needed here for tethering – I just am not sure if Grant and Rob will be specifically studying metabolism. Please advise.

Project #1: Physical substrates of systems biology

PIs: Jensen, Phillips

Christina Smolke 11/15/06 12:01 AM

Comment: Rob and Grant – I really need you to go through this project and clean it up – I essentially tried to structure what you sent me and cut and paste things you had written in. As a result it is no very well integrated and the flow is not good. However, this is the layout and format that it should be in. Please spend time going through this and flushing out the sections as requested.

Christina Smolke 11/15/06 12:02 AM

Comment: Should provide a brief overview of the project area and overall goals of the project and then go into a set of specific aims and what will be done under each specific aim as I have organized below. Please clean up as you feel appropriate.

A. Specific Aims

One of the main goals of systems biology is to dissect the interconnectedness networks and pathways that make cells tick. Often, these questions are posed in a way that features key informational properties such as the architecture of these networks. However, there is mounting evidence from many quarters that the physical properties of the molecules and assemblies that mediate cellular response can impact the biological function of these systems in intriguing ways. For example, it is becoming increasingly clear that nucleosomes are positioned on chromosomal DNA in a way that reflects the underlying mechanics of the DNA molecule. Similarly, some important signaling molecules have structures that involve floppy internal disordered domains and the length and mechanical properties of these internal domains can directly alter the signal readout from these signaling networks.

One of the unique threads of the work proposed here is that we aim to merge the informational outlook with an emphasis on the structure and physical properties of the underlying molecules and macromolecular assemblies that give rise to the observed system behavior. The unifying thread of all of this work is the construction of predictive, physical models and the design of experiments to test them.

Our goals for this will be to explore in several test cases how cells use structure to regulate the dynamics of system behavior. Specific aims include to:

1. **Determine the structure of the bacterial chromosome and the structural elements that organize it within the cell.**
   The experimental design will be organized as follows: **A.** Determine the structure of the bacterial chromosome using EM tomography; **B.** Develop statistical mechanical models to examine the role of tethering and confinement in dictating the arrangement of chromosomal DNA.
2. **Determine the architecture of the peptidoglycan layer and its connection to regulating cell shape.**
   The experimental design will be organized as follows: **A.** Identify the remaining cytoskeletal filaments within Caulobacter crescentus using EM tomography; **B.** Determine the most accurate model for peptidoglycan layer architecture by comparing proposed models to 3-D constructions of the EM tomography data; **C.** Develop and test hypotheses for how the observed cytoskeletal network governs cell shape.
3. **Explore the role of spatial control mechanisms such as tethering on the dynamics of network function.**
   The experimental design will be organized as follows: **A.** Solve the quarternary structure of a set of natural and synthetic tethered enzyme complexes using EM tomography; **B.** Examine how tethering impacts the dynamics of system behavior; **C.** Develop statistical mechanics models to examine how physical properties of the linker regions alters biological readout.

Christina Smolke 11/15/06 12:06 AM

Comment: It might be nice to start off with a brief intro or background to the general idea of structural regulating dynamics of cellular processes. Then go into background on each of the specific sub-areas as I list below. Feel free to modify the sub-titles that I put in and certainly the content and the flow. Try to make more cohesive. Can also include short background on both of your methods – EM tomography (or Grant maybe just reference the foundational technologies section for yours since there will be an overview in there) and perhaps the models. It is up to you but it should be brief.

B. Introduction and Background

DNA packing, structure, and function

Another example of the regulatory importance of structure is the packing of DNA within cells. It has recently been shown that even in bacteria, the genome is highly organized, with the

spatial arrangement of genes within the cell matching their linear arrangement in the primary sequence of the chromosome. What structures within the cell pack and organize the chromosome, and how it is segregated during cell division, is still entirely unknown. In the case of at least one sporulation gene, moving it from one position within the circular chromosome to another altered its function.

One class of problem of particular interest where DNA deformation is explicitly tied to cellular decision making are those involving large scale DNA loops. There are a number of examples of cellular decision making that apparently involve some random choice. For example, in the context of yeast, the mating type switch involves a recombination event where two distinct chromosomal modules are required. Similarly, the selection of olfactory receptors involves the formation of large DNA loops. Finally, the development of antibodies in the immune system also involves recombination events that require distant elements on chromosomes to be brought into close physical proximity.

Cytoskeleton, peptidoglycan, and cell shape

The advent of cryo-electron tomography has literally required that we rewrite the textbooks on some topics such as the structure of mitochondria. Further, in some cases, these new techniques are bringing structures into view that have until now been completely unknown. The discovery of such structures is in keeping with one of the overall themes of the proposal which is the idea of dissecting how the physical substrates of systems biology control and alter biological function. Increasingly, these structural experiments have graduated from an era that is strictly observational to one in which it is becoming possible to pose precise, quantitative questions. One class of questions that are of particular interest have to do with the molecules and forces that dictate the shapes of cells and their organelles.

Bacterial cells come in a wide variety of shapes and sizes: coccoid, rod-shaped, spiral, square, and star-like. How and why each species generates and maintains its particular shape is still unknown, but it has become clear that certain cytoskeletal filaments and the cell wall are key determinants. MreB is a prokaryotic actin homolog which appears to be crucial for rod shapes and the establishment of polarity. FtsZ is a tubulin homolog that forms a cytokinetic ring at the division plane. Crescentin is an intermediate filament-like protein necessary for the crescent shape of Caulobacter cells. Nevertheless it is believed that the cell wall, and more specifically the peptidoglycan layer, is actually the structural element that resists deformative forces. The cytoskeletal filaments act principally by positioning peptidoglycan synthases, directing the shape of that layer as it is built.

The architecture of the peptidoglycan layer is unclear. Peptidoglycan in bacterial cell walls is a fascinating, giant molecule made up of sugars and peptide linkages. While its chemical building blocks are well-characterized, it is not known whether individual peptidoglycan strands lie normal to the cell surface or tangentially, how tightly they are packed, how they are spaced, or how they are added to allow the cell to grow. Some models have been proposed and one of the specific aims of this project is to dissect the complex orchestration of cell shape in bacteria.

Protein tethering, spatial organization, and function

One of the ways that structure influences “systems biology” is by organizing enzymes within the cell. These spatial relationships can have dramatic effects on dynamics (i.e. the flux through the metabolic pathway). There are many degrees of organization. Sometimes enzymes are grouped by containers like membranes or protein shells. Carboxysomes are particularly

interesting examples of the latter. These organelle-like structures concentrate RuBisCO and its CO2 substrate within many autotrophic bacteria. Another strategy to organize enzymes is to simply tether them together. The pyruvate dehydrogenase multienzyme complex (PDMC) is an interesting example of tethering. There are five separate reactions required to convert pyruvate to acetyl-CoA, catalyzed by a set of three different proteins. In some organisms, these three proteins are released individually into the cytoplasm, and the flux through the pathway depends on substrates and enzymes colliding during the course of random diffusion. In more advanced cells like *E. coli*, the three proteins are linked together by tethers. In even higher organisms, another strategy is used, which is to bind the proteins together into rigid complexes. In mammalian cells, for example, the pyruvate dehydrogenase multienzyme complex is icosahedral, leading to efficient substrate channeling.

One of the intriguing physical tricks that cells have evolved to more tightly control network properties is the use of tethering. The basic idea is that two different domains are linked together, either by an intervening disordered protein region or by DNA. Two key examples of interest here are: pyruvate dehydrogenase, an enzyme relevant to the metabolic pathways of cells and N-Wasp, a protein that plays an important role in dictating when and where actin polymerization will take place. In both of these cases, one of the most compelling structural features of these molecules is that they have physical tethers that link different parts of the molecule.

C. Preliminary Results

Christina Smolke 11/15/06 12:04 AM
Comment: I need preliminary results in each one of the specific aims from both you and Rob. Please get these in here including any figures, etc. Should be brief.

Specific Aim #1: Bacterial chromosome structure and its organization within the cell

Electron cryotomography produced three-dimensional reconstructions of whole cells in an intact, near-native state at "molecular" resolution (~5 nm). In some cases the cytoplasm within these reconstructions show two distinct regions with different textures. One region is full of large particles that are almost certainly ribosomes. The other region excludes ribosomes, and is probably the genome-containing nucleoid. In the most favorable cases, we have now been able to detect linear elements within these nucleoids, giving what is likely the first glimpse of the chromosome structure in its native state.

Specific Aim #2: The architecture of the peptidoglycan later and its relation to cell shape

The Jensen group has recently begun recording three-dimensional images of whole bacterial cells with "molecular" resolution (~5 nm). So far this work has targeted Magnetospirillum, Caulobacter, Treponema, and Mycoplasma species. In the case of Caulobacter, the only bacterium known to contain representatives of all three major classes of cytoskeletal filaments, six distinct types of filaments bundles were observed in different locations within cells and different stages of the cell cycle. In an attempt to identify the different bundles, overexpression, depletion, and deletion mutants have been imaged. In the case of FtsZ, an overexpression mutant has now revealed the structure of the FtsZ ring.

The Jensen group has also obtained the first 3-D structures of the peptidoglycan layer in a native state.

Specific Aim #3: The relation between spatial control mechanisms and system dynamics

In pyruvate dehydrogenase, the three proteins catalzying the five reactions are named E1, E2, and E3. Following decades of study, atomic models for all the ordered domains of E1, E2, and E3 were available, but the quaternary structure was not. We showed by direct imaging that the E1 and E3 subunits of these complexes are flexibly tethered ~11 nm away from the E2 core (ref).

This result demonstrated that electron cryotomography could reveal the relative positions of features as small as 80 kDa in *individual* complexes, elucidating quaternary structure and conformational flexibility.

Recent work in the Phillips group has exploited statistical mechanics to work out how the physical properties such as length and stiffness of these linker regions alters the biological readout.

D. Research Plan

Christina Smolke 11/15/06 12:25 AM

Comment: Again, please detail BRIEFLY what you plan to do under each specific aim. Please feel free to change specific aims as appropriate and provide a description as best you can of what you will do. I cut and paste text from what I had, but it definently needs to be gone over, edited, and added to.

Specific Aim #1: Bacterial chromosome structure and its organization within the cell

A. Determination of structure of the bacterial chromosome and the structural elements that organize it within the cell

Intact bacterial cells will be imaged in 3-D by electron cryotomography to visualize the structure of the bacterial chromosome. Growth conditions will be found that alter the structure of the nucleoid, providing more clues about its dynamic properties. In order to increase the resolution and interpretability of the reconstructions, toxins will be added that produce small holes in the cell wall. These holes will allow most cytoplasmic contents to "leak" out of the cell, yielding better imaging conditions to visualize the chromosome. Finally, sophisticated image processing routines will be applied to identify all linear elements within the nucleiod.

B. Development of statistical mechanical models to examine the role of tethering and confinement in dictating the arrangement of chromosomal DNA

We propose to use statistical mechanical models to explore the physical forces that shape chromosome geography and help dictate stochastic decision making in these important contexts. In particular, we will extend our work on short distance looping to consider the in vivo looping properties of DNA. In particular, we (in collaboration with Professor Jané Kondev of Brandeis University) have been engaged in using models of chromosome geography based on the random-walk picture of chromosomal DNA. The novel features of these approaches are to examine the roles of tethering and confinement in dictating the arrangement of chromosomal DNA. Further, depending upon the state of compaction of the chromosomal DNA (i.e. 30 nm fiber), the persistence length of this chromosomal DNA will be different and this too is one of the parameters that enters our theoretical description.

Specific Aim #2: The architecture of the peptidoglycan later and its relation to cell shape

A. Identification of the remaining cytoskeletal filaments within Caulobacter crescentus

Three main strategies will be employed: (1) imaging expression mutants of candidate cytoskeletal proteins, (2) correlated light and electron microscopy, and (3) sensitive computational searches for filaments.

B. Determination of the most accurate model for peptidoglycan layer architecture

Psuedo-atomic models of the two main candidate architectures of the peptidoglycan layer (peptidoglycan strands normal and tangential to the cell surface) will be constructed. These models will be compared by several quantitative measures to experimental densities in 3-D reconstructions of whole cells in their native states.

As a result of the recent cryo-electron microscopy studies in the Jensen lab, we have been engaged in an attempt to make a more precise quantitative census of an individual bacterium. One of the prerequisites to real cell physiology (or systems biology) is to have a precise

reckoning of the various molecules that make up the network of interest. Indeed, one of the outcomes of the many proteomic studies is counts of many of the soluble proteins in cells as a function of perturbations that the cell has been subjected to. Unfortunately, carrying out such a census on the membrane/cell wall part of cells is much less routine. Part of that census focuses on the molecular mass inventory of the cell wall and we are developing simple physical models of the peptidoglycan network and explicitly testing those models against the cryo-electron microscopy results coming out of the Jensen laboratory. In particular, we propose to develop automated methods for analyzing cryo-EM images of the cell wall which will yield the thickness and density of the peptidoglycan. These experimental images will be compared with simulated images based on real molecular models of the cell wall material.

C. Development and testing of hypotheses for how cytoskeletal network governs cell shape

Spatially-explicit simulations will be run wherein peptidoglycan synthetic enzymes will be positioned along the observed cytoskeletal network and tracked as they add peptidoglycan strands to a growing peptidoglycan layer. These simulations will test the plausibility of different ideas about how the cytoskeletal network might be governing cell shape.

Specific Aim #3: The relation between spatial control mechanisms and system dynamics

A. Structural determination of a set of tethered enzyme complexes

B. Examination of the effects of tethering on the dynamics of system behavior

C. Development of statistical mechanics models to examine the relation between physical properties of the linker regions and biological readouts

E. Challenges and Alternatives

References

Christina Smolke 11/15/06 12:26 AM
Comment: This is part of the criteria by which they will judge the merit of the proposal – please try and consider particular challenges with what you are proposing and different alternative strategies to those challenges.

Christina Smolke 11/15/06 12:27 AM
Comment: Please go through and include these to make project more detailed and specific. Try to target for length (minus references) for app. 10 pages ish.

Project #2: Control architectures regulating flux through metabolic networks
PIs: Smolke, Jensen

Christina Smolke 11/15/06 12:17 AM
Comment: I will work on this write up in the next couple of days.

Project #3: Global transcriptional regulatory architectures and their effects on population heterogeneity

PIs: Elowitz

Christina Smolke 11/15/06 12:19 AM
Comment: Michael, I have not made changes to the formatting since you mentioned you will be sending a new version. I am including this so Rob and Grant can see your proposal. Please check this title, think about it, and propose a change as you like.

Christina Smolke 11/15/06 12:20 AM
Comment: Michael – all of the other projects in this proposal have multiple PIs on them. Is there any need, benefit, desire to integrate any one else on this proposal. It is up to you but you may want to think about – anyone else have ideas on if they can connect any of their stuff to this proposal?

Specific Aims

A substantial amount of information is now available on the global regulatory architecture of *B. subtilis*, as well as the regulatory structure of particular stress responses. At the same time, it has become clear that individual *B. subtilis* cells exhibit enormous heterogeneity even in uniform environmental conditions. Thus, a fundamental question is how the regulatory architecture of the cell gives rise to the distribution of cellular states observed in various conditions. The specific aims of this research are to answer this question through a comprehensive approach combining experiments at the single-cell level and theoretical modeling.

Aim 1: Construction of a 'matrix' of strains, each containing three distinguishable fluorescent reporters for specific cellular regulators.

Based on the known architecture of the cell, we will identify a set of promoters that reflect key regulatory activities in the cell. Using flow cytometry we will undertake an initial screen of an array of transcriptional regulators chosen for their predicted heterogeneity, under several environmental conditions. We will select approximately ten promoters with quantifiable levels of expression, substantial variability, and broad distribution across global stress responses for further analysis. Using these promoters, we will construct a "matrix" of strains containing combinations of 3 different fluorescent protein promoter fusions for further in-depth analysis.

Aim 2: Correlation analysis of the reporter strain matrix.

We will characterize the static and dynamic features of the interactions among different stress response modules. We will analyze the strain matrix constructed in Aim 1 using both multi-color flow cytometry and time-lapse microscopy analysis to analyze static and dynamic correlations, respectively. Based on the static results we will choose a subset of environments and reporters for further analysis of their dynamical behavior. We will also create a set of mixed environmental conditions designed to activate stress response combinations. These will be used to identify environmental regimes with enhanced inter-modular interactions. Finally, all correlations will be analyzed in the context of mathematical models of known features of the underlying genetic circuitry.

Aim 3: Perturbation analysis of the reporter strain matrix.

In Aim 3 we will apply forward experimental approaches to better understand the correlations revealed in Aim 2. Specifically, we will test hypotheses of specific dynamic gene expression mechanisms using genetic perturbations. The approach involves constructing stains regulatory gene expression can be perturbed in several ways. First, we will modify natural genes to allow tunable downregulation. Second, we will insert extra copies of regulatory genes under inducible promoters. Finally, we will insert extra copies of regulatory genes under the control of copies of different natural promoters with known regulatory patterns. This latter, 'rewiring' approach will enable specific manipulation of circuit connections. The resulting perturbation

strains will be analyzed by flow cytometry and time-lapse fluorescence microscopy. Data will be analyzed in the context of mathematical models of the corresponding regulatory circuits.

Introduction: the global regulatory structure of the cell.

Complex cellular behaviors are controlled by circuits composed of interacting genes and proteins. Although many components and interactions in these circuits have been identified, it remains unclear how they function dynamically at the single cell level. In particular, genetic circuits exhibit substantial fluctuations, or noise, in their components. Noise is a fundamental aspect of life at the single cell level that can arise from intrinsic stochasticity in intracellular biochemical reactions. Recently noise has been shown to be particularly significant in gene expression [Elowitz2002]. By reducing the precision of these circuits, noise can disrupt their function. At the same time, noise has been shown to facilitate the generation of heterogeneity, including multiple differentiated cell types, in isogenic cell populations.

These observations raise fundamental questions about the interaction of noise and the global regulatory architecture of the cell: How uniform are cellular states? Does noise propagate through global regulatory networks, and do cellular circuits limit this propagation? To what extent does the response of cells to noise mimic their response to changes in environmental conditions?

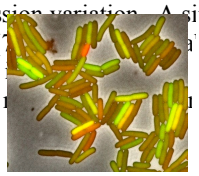
This study will address these questions using a combination of experimental and theoretical techniques. Using multiple colors of fluorescent protein reporters, it is now possible to determine the expression patterns of various responses directly in individual living cells. The soil bacteria *Bacillus subtilis* exhibits a broad range of heterogeneous behaviors, making it the ideal model organism for this work. Our approach is based on the construction of a comprehensive set of strains, each containing overlapping sets of up to three fluorescent reporters. We will analyze these strains using quantitative time-lapse fluorescence microscopy and automated image analysis techniques that we developed previously. These methods, together with mathematical modeling of cellular circuitry, will allow us to focus on three Aims: First, we will create a set of bacterial strains characterize correlations among the activities of global regulatory genes to determine the independence or crosstalk among various regulatory responses. Second, we will analyze the causal basis for these correlations by perturbing (increasing or reducing) specific activities of key regulators and observing the responses of other regulators. Third, we will use the data from the first two Aims to construct a mathematical model that accounts for temporal correlations among key regulatory genes. Results from this model will be immediately testable using the techniques of the first two Aims, enabling iterative refinement.

Together, these results will enable us to analyze how fluctuations, together with the regulatory architecture of the cell, determine the spectrum of possible cellular states. They should in addition help us to understand the functional significance of the global architecture of *B. subtilis.*

Background and Significance

Noise at the single-cell level.

Noise in gene expression. Intracellular biochemical reactions involve substantial stochasticity, or 'noise', but this variation is hidden by traditional methods that average over large cell populations (for reviews on noise, see [1-3]). Recently, the magnitude and mechanism of noise generation in bacterial gene expression have yielded to approaches based on fluorescent protein expression [4-6]. For example, using two distinguishable fluorescent protein reporter genes under the control of identical promoters we showed that variation in expression of a particular gene can be divided into two components: 'intrinsic' stochasticity in gene expression, and upstream, or 'extrinsic' noise due to fluctuations in transcription factors or other cellular components (see Fig. 1) [4]. The results showed that intrinsic stochastic noise can be the dominant source of gene expression variation. A similar technique has now been used to analyze gene expression noise in yeast [7] -cell fluctuations is even larger. We note that variability d functionally important [10] in mammalian cells. Thus, ol of gene expression at many biological levels.



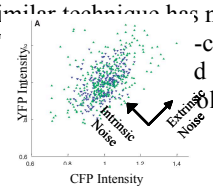


Figure 1. Noise in gene expression. (A). *E. coli* cells with CFP (green) and YFP (red) fluorescent proteins expressed from identical promoters. Color variation indicates the level of intrinsic noise. Without noise, all cells would appear yellow (equal amounts of red and green). (B) Analysis of noise. Each dot represents a single cell from (A) in CFP (x-axis) and YFP (y-axis). Without intrinsic noise, all points would lie on the diagonal line. Additional variation parallel to this line represents upstream, or 'extrinsic', noise in other cellular components.

Dynamics of noise. Noise has a timescale as well as amplitude. While very fast fluctuations may quickly 'average out' and thus have no effect on a genetic circuit, slow fluctuations can fundamentally limit the accuracy of biochemical processes. We recently analyzed a synthetic repressor 'cascade' to determine how repressor concentration and noise together affect target gene expression rate [11]. As shown in Fig. 2, a fusion protein consisting of lambda repressor fused to YFP, was allowed to dilute out in a growing *E. coli* microcolony. These cells also contained a *cfp* target gene. Because we used time-lapse microscopy, the rate of expression of *cfp*, and its fluctuations, could be determined as a function of repressor concentration in individual cell lineages. After controlling for the transcription factor itself, we found that most fluctuations were extrinsic in origin and slow (i.e. they fluctuate on a timescale of one cell cycle). Because of their slow speed, they cannot not be 'averaged out' by the cell, and this limits the accuracy of simple gene regulation [11].

Noise in differentiation processes. Because of the regulatory structure of genetic circuits, intrinsic noise in the expression of, say, a transcription factor, can cause extrinsic noise in the expression of its targets; i.e. noise can propagate through genetic circuits [6, 11-13]. Experimental work has analyzed the effects of negative feedback on noise [14], and the propagation of noise through a cascade [12], in designed constructs (synthetic genetic circuits). In a more natural context, the effects of multiple feedbacks on induction of metabolic genes were analyzed both experimentally and theoretically in yeast [15]. Noise has also been examined in the developmental context of anterior-posterior patterning in *Drosophila.* In this system, evidence for noise, and its suppression, was recently described [16]. Nevertheless, in general it has remained unclear how noise is actively managed or suppressed by developmental networks which make cell-fate decisions and carry out robust developmental programs.

Random decision-making. A number of bacterial systems have been shown to make effectively random decisions under some conditions [1]. A medically important example is the persistence phenotype: individual cells stochastically enter a slower growing state in which they can transiently withstand otherwise lethal antibiotic treatments [17, 18]. Recently, such 'bet-hedging' strategies have been shown theoretically to convey advantages over deterministic strategies [19-21]. Some of these strategies are implemented with DNA rearrangements but many of them are implemented by genetic circuits. In such circuits, features such as positive feedback loops that can stabilize alternate states are expected theoretically, and often found. However, it has remained unclear how natural gene circuits allow cells to control the frequencies with which they differentiate. Secondly, although noise inherent in cellular processes is likely to be involved, the underlying 'coin-flipping' mechanism cells use to make random decisions remains unclear. Most importantly, it remains unclear how fluctuations in diverse response pathways are coordinated either to prevent cells from entering inappropriate states, or simply to channel variation into preferred states.

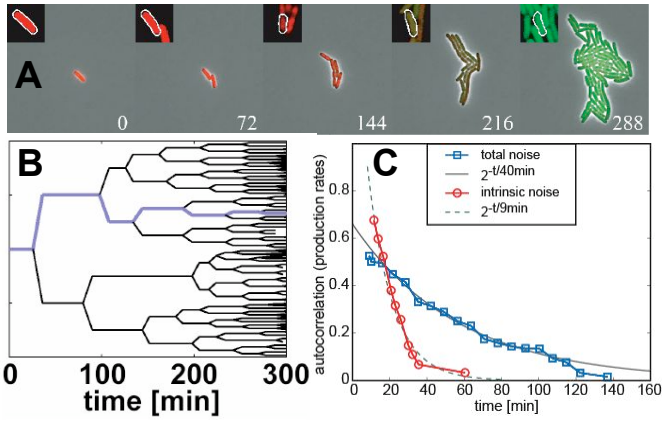


Figure 2. Example of time-lapse analysis of gene regulation and noise at the single-cell level. (A) Movie of a growing *E. coli* microcolony, in which a repressor (red fluorescence) is diluted out during growth, so that its target gene (green) is eventually expressed. Insets show an individual cell identified by tracking software. (B) Tracking software produces a lineage tree from the movie in (A), with selected lineage highlighted in blue. (C) Autocorrelation functions based in part on data in (B). These plots show that intrinsic noise fluctuates rapidly, but extrinsic noise fluctuates with a slow time-scale of one cell cycle (40 min. in these conditions).

Why B. subtilis*?* There are numerous developmental systems in which noise could be studied, including stem cell differentiation, fly development, and yeast stress response. However, *B. subtilis* has a unique and powerful combination of features that ideally suit it to this problem: First, *B. subtilis* exhibits a range of alternative states and stress responses, such as chemotaxis, competence, sporulation, chaining, biofilm formation, and many others, regulated by the largest combination of well-characterized and diverse sigma and transcription factors. Gene circuitry surrounding these responses is, in many cases, well-characterized. This will enable us to focus immediately on known genes and interactions. Second, imaging in bacteria can be extremely quantitative, as we have shown in previous work [4, 11], enabling us to directly compare models with the expression dynamics of genes within the system. Third, *B. subtilis* can be very easily modified genetically, allowing us to rapidly test specific predictions and vary quantitative circuit parameters at will. These features enable us to directly connect molecular noise in individual cellular components with phenotypic variability using mathematical models.

Key Techniques

Over the last five years we have acquired significant experience with the techniques needed for the proposed research:

Technique 1. Automated time-lapse microscopy: We have developed a custom software/hardware system that can acquire ~20 simultaneous movies of growing microcolonies with automatic focusing and temperature control. The acquisition software allows for different settings for different movies, and thus offers both flexibility and high throughput. Multiple conditions can be probed simultaneously using individual agarose pads mounted on the same dish (Fig. 3). This technology was initially developed for synthetic experiments [22] but has been much improved [11]. We now have 3 acquisition systems operating daily in the laboratory.

Technique 2. Quantitative Image analysis: One can acquire time-lapse image data much faster than one can analyze it. For the experiments proposed here, accurate and efficient quantitation of fluorescent protein expression in individual cells is critical. We have developed a custom software package ('Schnitzcells') that detects cells in phase contrast images and tracks them over time (Fig. 3). The output of the software is a lineage tree for the growing microcolony (Fig. 2), and fluorescence information for each cell and time-point. In ref. [11], we used Schnitzcells to track *E. coli* microcolonies. We have since modified the software to detect *B. subtilis* spores and forespores automatically (not shown).

Technique 3. Mathematical modeling: We have developed substantial expertise in the mathematical modeling of genetic circuits. This was first used to make qualitative predictions about possible oscillator mechanisms [22]. Later we modeled noise production and propagation in a synthetic cascade [11]. As shown below (C.1 and D.2), we are applying such models to the competence system, and developing similar models for sporulation initiation. We also benefit from the expertise of collaborator Jordi Garcia-Ojalvo (see attached letter of collaboration?)

Technique 4. *B. subtilis* molecular genetics and strain construction: We have developed a PCR-based system for rapid assembly of *B. subtilis* strains containing up to three fluorescent protein reporter genes. The scheme uses fusion PCR and a set of modular integration vectors (based on

those provided by the *Bacillus* stock center*)*. As shown in Fig. 3B, we find that expression of fluorescent reporter genes is independent of the location of their respective integration sites within the *B. subtilis* chromosome. Our collaborator, Jonathan Dworkin, has extensive expertise in other genetic manipulations in this organism [23-27].

Technique 5. Optimized media conditions and protocol for observation of dynamic fluctuations in stress response systems. Ideal growth conditions allow several generations of growth (enough so that one can observe fluctuations over several cell cycles, together with lineage relationships, but not so many that cells become confluent or 'pile up' on the slide). We have developed a relatively simple protocol, involving transfer of growing cells to *B. subtilis* resuspension medium [28] that reproducibly meets these criteria [29]. We have also used other, rich, media conditions to observe gene expression dynamics during *B. subtilis* growth. Finally, a specific issue that arises with *B. subtilis* is that during rapid growth cells tend to "chain" into long filaments. We have addressed in more recent work, by obtaining and analyzing movies at lower magnification, while maintaining good image analysis results [In progress].

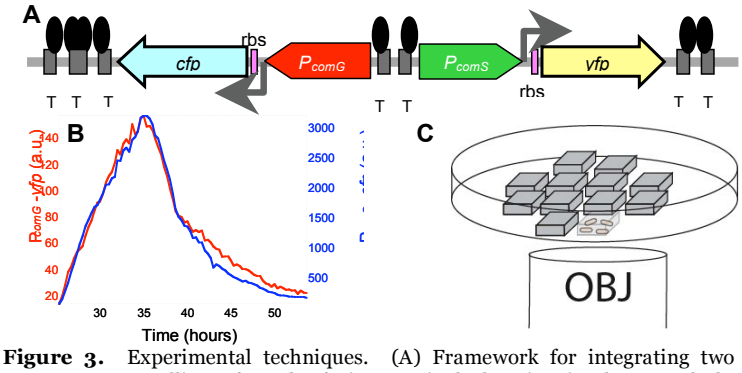


Figure 3. Experimental techniques. (A) Framework for integrating two promoters controlling *cfp* and *yfp* into a single location in the *B. subtilis* chromosome. Other promoters can be rapidly substituted for P_{comG} and P_{comS} by PCR. (B) Similar expression of CFP and YFP obtained from identical promoters (P_{comG}) at different loci. This demonstrates that maturation and degradation kinetics are equivalent for the two reporters, and that different integration loci are equivalent (not true during asymmetric septation—see Fig. B.3.2.). Note that intrinsic noise is relatively small due to strong expression. (C) Experimental setup for simultaneous recording of cells under multiple conditions. Agarose slabs (gray) cover cells (shown in the front-right). The bottom surface of the dish is a coverslip.

Research design and methods:

1. Construction of an overlapping multi-color strain set (Aim 1)

The regulatory circuitry of *B. subtilis* is controlled by a large number of interconnected transcription factors (Figure 1), including many alternative sigma factors [30]. We will focus this study on transcriptional regulators that are well-characterized, that are activated in media conditions that we will analyze, and that show significant cell-cell variability. A further condition is that the promoter set should be as diverse as possible while compact enough, at least initially, to allow comprehensive analysis.

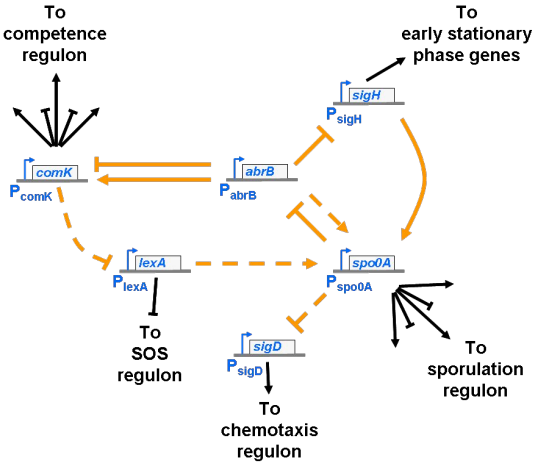


Figure 4. Simplified network diagram for subsection of global regulators (For clarity only gene promoters depicted, dotted lines represent indirect repression or activation). ComK, the master transcriptional regulator for competence, indirectly inactivates the repression of the SOS operon by LexA [31] through the activation of RecA (not shown). LexA indirectly activates *spo0A* expression, by repressing *sdA* (not shown), a known inhibitor of *spo0A* phosphorylation [32]. Phosphorylated Spo0A directly represses *abrB* [33], causing upregulation in σ^H expression. Spo0A has also been shown to repress chemotaxis [34]. σ^H activates many early stationary phase genes, including *spo0A* [35]. AbrB has been shown to both activate and repress *comK*, and indirectly activates *spo0A* expression.

Construction of the strain set will proceed in several steps:

Step 1: Selection of genes. Based on the literature we will identify approximately candidate regulatory genes and corresponding target promoters that can act as activity reporters. As an example, an initial list of 10 key transcriptional regulators is shown in Table 1. Each promoter will be fused to a *yfp* reporter gene and integrated into the chromosome of a strain containing the $P_{\sigma A}$-*cfp* reporter gene as reference. Each promoter will be scored by flow cytometry for mean and variability of expression level in several media conditions (Table 2). We will then select a set of 9 most promising promoters for further study. This number N=9 is chosen for convenience, to permit analysis of the 3-color matrix, with its $\binom{N}{3}$ unique combinations of promoters (for N=9, there are 84 combinations). The 'diagonal' elements in which the same promoter is used to control multiple colors are useful for determining the origins of noise (intrinsic stochastic processes in gene expression vs. extrinsic effects due to regulators or other fluctuations), as we have done previously [4].

Step 2: Multi-color promoter matrix. Once a suitable initial set of approximately 10 promoters has been identified, we will construct a "matrix" of strains containing sufficient, but not exhaustive, promoter combinations. Because of the modular design of the promoters, and the ability to cross *B. subtilis* strains rapidly, it will be possible to rapidly generate arbitrary strains from the matrix within a timescale of 1-2 weeks, and in parallel. To facilitate this, each fluorescent protein reporter will be associated with a single chromosomal integration site and antibiotic resistance marker.

Table 1. List of *B. subtilis* promoters for analysis.

Transcriptional Regulator	Function	Target promoter	Inducing conditions	Reference
σ^A	"Housekeeping"	**$P_{hyperspank}$**	All	
Spo0A	Sporulation Initiation	P_{spo0A} P_{spo0F}	Nutrient limitation	[36]
σ^D	Chemotaxis	P_{sigD} P_{hag}	Vegetative growth **(?)**	[37]
σ^B	General Stress Response	P_{sigB} P_{gsiB}	Include heat shock, salt stress, ethanol, starvation	[38-40]
σ^H	expression of early stationary-phase genes	P_{spo0A} P_{lytE}	Nutrient limitation	[41, 42]
AbrB	Regulator of early stationary-phase genes	P_{abrB}	Vegetative growth **(?)**	[43-45]
ComK	Competence Initiation	P_{comK}	Nutrient Limitation	[46]
LexA	SOS regulon	P_{lexA}	Repressed by DNA damage agents	[47]
CcpA	Catabolite repression	P_{ackA}	Glucose	[48]
TnrA	Nitrogen regulation	P_{tnrA} P_{alsT}	Nitrogen Limitation	[49]

Table 2. List of media conditions for analysis of correlations.

Conditon	Induced promoters	Reference
"Resuspension Media"	P_{comK}, P_{spo0A}	[28]
Salt Stress	P_{sigB}	[50]
Heat Shock	P_{sigB}	[51]
UV radiation	P_{lexA}	[47]
Nitrogen Limitation	P_{tnrA}	[52]
High Glucose	P_{ackA}	[53]

2. Correlation analysis of the multiple reporter strain (Aim 2)

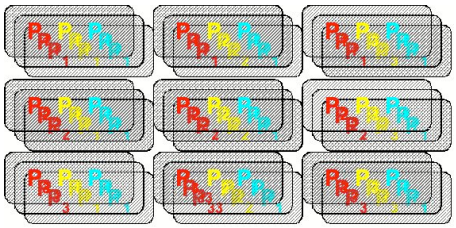


Figure 5. Three-color strain matrix (schematic). The promoters, P_1, … P_N, are chosen from a list of characterized target promoters (Table 1). Colors indicate RFP (red), YFP (yellow), and CFP (cyan). Each strain in the matrix can be easily constructed from 3*N* single-color strains. Note that not all of the strains in the 3-dimensional matrix need to be constructed (see text).

A. Analysis of correlation in "pure" environments.

A fundamental aspect of the physiology of the cell is the degree of correlation among its various responses. Having characterized heterogeneity in the stress response promoters described above (Aim 1), and constructed the strain matrix (Aim 1), we will characterize these correlations at several levels:

Step 1. Flow cytometry analysis of the strain matrix. We will use flow-cytometry (based on a convenient bench-top Beckman-Coulter Quanta analyzer in the lab) to analyze the strain matrix in each of the environments listed in Table 2. These environments are chosen to preferentially activate specific stress responses. Analysis of this data set will allow us to quantify relationships between the different stress modules at the single-cell level. These data will directly reveal the degree of coupling among stress responses.

Step 2. Analysis of the strain matrix in mixed environments. In many cases, we expect the relatively "pure" stresses analyzed above (Step 1), to show only minor induction of other stress responses. Hence, in step 2, we will analyze the interaction between stress reporters in mixed-stress environments. Mixed conditions will reveal subtle interactions between the different modules. As an example we will analyze the interaction between sporulation and general stress modules under nutrient limitation and high salt conditions. To that aim, candidate reporter strains will be studied in the context of an environmental matrix with varying degree of each stress on the different axis.

Step 3: *Dynamical analysis of module interaction.* Based on the results of steps 1 and 2 we will select a narrower set of reporter strains and corresponding environments for further analysis using time-lapse microscopy. This will allow us to observe dynamical aspects of the interaction on a single cell level and answer questions regarding the dynamics of switching or cooperation between stress responses. Our previous time-lapse studies have shown that powerful quantitative information can be obtained by directly analyzing the quantitative responses of multiple elements in the same cell. For example, in [rosenfeld2005] we discovered that cellular fluctuations in the rate of gene expression have a slow autocorrelation time that limits the accuracy of transcriptional regulation. Similarly, in [Suel2006] anti-correlation between the *comS* and *comG* promoters in individual *B. subtilis* cells provided evidence for a negative

regulatory interaction. Here, we will use the simultaneous temporal response profiles of several reporter genes to similarly infer timescales of fluctuation and possible interactions.

Possible pitfalls: A major source of concern is the ability to use flow-cytometry to simultaneously observe three colors with a wide dynamical range. In the case it is not possible, we will use fluorescence microscopy for steps 1 and 2 as well as e. This will reduce the number of the cells analyzed, but will increase the sensitivity of the system.

Aim 3: Response to perturbations

The correlation experiments above can suggest strong hypotheses about regulatory connections among system elements. However, to establish the causality of these hypothetical connections, we will need to perform controlled perturbations on its elements. These perturbations fall naturally into two categories:

1. *Over-expression, deletion, and tunable repression.*

Simple genetic perturbations such as over-expression or deletion provide substantial insight into gene function by altering phenotype. Established methods exist to generate these perturbations in *B. subtilis.* Over-expression constructs can use a variety of inducible promoters which function effectively in *B.* subtilis, responding to different inducers such as IPTG and xylose (Fig. 7). These promoters possess both tight regulation and substantial dynamic range. Deletion constructs can also be easily generated through homologous recombination.

We are also currently exploring a simple method to tunably repress endogeneous gene expression (Fig. 6). This method modifies wild type promoters by adding one or more lacI binding sites between the -8 and +13 bases relative to transcription start site, causing a tunable repressive effect. Native promoters are replaced in their wild-type locations with the engineered version using homologous recombination. This method preserves the native promoter regulation, while varying IPTG amounts tunes promoter activation from wild-type (at full IPTG induction) to fully repressed (no IPTG).

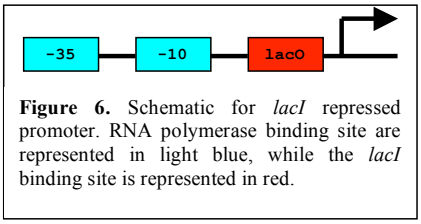


Figure 6. Schematic for *lacI* repressed promoter. RNA polymerase binding site are represented in light blue, while the *lacI* binding site is represented in red.

2. *Re-wiring*

Another, more subtle, type of perturbation places endogenous genes under the control of alternative promoters. Genes under the control of these alternative promoters can experience regulation dynamics markedly different from the wild type. This method allows us to take advantage of the rich dynamics of one circuit to perturb the functioning of another. The screens from Aim 1 and Aim 2 should reveal a wide variety of gene expression dynamics and noise profiles to use for these modifications. We describe networks modified in this fashion as "rewired." As described below, we have successfully applied this method to analyze the *B. subtilis* competence network. In that case, the sudden turn-on of the competence system could be used to rapidly switch on an inhibitory gene, to determine the effect of a strengthened negative

feedback on competence dynamics (Fig. 8). The same technique was also used to demonstrate the necessity of negative regulation of *comS* for competence exit [Suel2006].

Looking forward, our initial screen of genes has yielded several attractive candidates for rewiring of the global stress response network. To take one example, we have identified endogenous oscillatory dynamics in the transition state regulator *abrB* (shown below). This promoter can potentially drive various genes with oscillatory dynamics. Known interactions surrounding the sporulation initiation circuit provide a variety of other interesting candidates for rewiring. For example, previous work has demonstrated that sporulation initiation genes are activated and/or repressed at a wide variety of spo0A levels [Fujita]. These promoters can be used to activate or repress other transcription factors at specific stages of sporulation initiation.

Preliminary data

1. Construction of three-color promoter strains (Aim 1)

We have constructed several multi-color reporter strains (Table 3) in order to test the feasibility of our approach. We have verified that these promoters function correctly, and that the inclusion of multiple reporters does not disrupt the activity profile of individual promoters.

Table 3: Constructed reporter strains

Strain Number	CFP promoter	YFP promoter	RFP promoter
1	P_{spo0A}	$-P_{abrB}$	$-P_{sigmaH}$
2	P_{σ^D}	P_{spo0A}	none
3	$-P_{abrB}$	P_{spo0E}	P_{spo0A}
4	P_{comG}	P_{abrB}	P_{spo0A}

Correlation analysis of multiple reporter strains (Aim 2).

To verify the possibility of co-observing two stress responses, we used a marker for genetic competence (P_{comG}-*yfp*) together with a marker for the initiation of sporulation (P_{spo0A}-*rfp*). A strain carrying these two markers was stressed by growth to stationary phase followed by resuspension in standard sporulation media. A time-lapse movie was obtained by putting the cells on an agarose pad and observing them every 20 minutes with phase-contrast and fluorescence microscopy for 3 days. While most of the cells followed an asynchronous path to sporulation, a subpopulation became competent (~3% of cells). Analysis of the movie indicates that competent cells halt *spo0A* expression at a constant level during the transient competence event, and then resume sporulation.

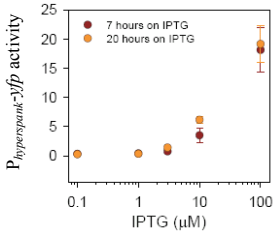


Figure 7. Calibration of $P_{hyperspank}$-YFP transcription in response to IPTG induction. This shows that a wide range of activation levels can be consistently accessed using IPTG induction.

This example shows the accumulation of knowledge as we progress through Aims 1 and 2 for a single strain. Fig. XXXA shows an artificial single color cell-sorter results at several stages of the movie – from these results we can understand that the system shows large heterogeneity (with a bimodal distribution) in

the two stress responses. Figure XXXB shows the same stages but now with a two colors correlation snapshot view of the stress modules – here it can be seen that the states are mutually exclusive.

Aim 3: Response to perturbations

Over-expression and deletion. To probe the characteristics of our inducible promoters, we have engineered strains containing a $P_{hyperspank}$-YFP construct, where the IPTG inducible hyperspank promoter expresses YFP. Gene expression is induced under microscopy conditions by adding suitable concentrations of IPTG to agarose pads covering cells (Fig. 3C). IPTG expression is tunable over a large dynamic range, making it suitable to perturb a wide variety of genetic circuits (Fig. 7). Furthermore, the circuit is tightly repressed at low IPTG levels, allowing a valid comparison with wild-type.

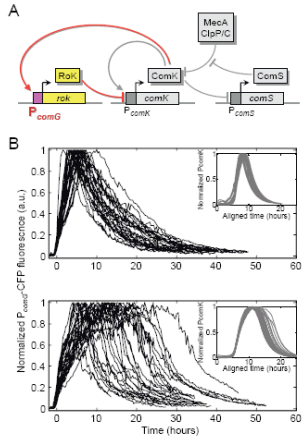


Figure 8. Response of competence to rewiring by insertion of an artificial P_{comG}-*rok* operon. (A) Network diagram with rewiring modifications shown in yellow. (B) Competence events with (above) and without (below) rewiring modification. Note the reduced duration of competence.

Re-wiring. We demonstrated the potential of the rewiring method in our earlier study on genetic competence [Suel2006]. In that system, placing the competence regulator *comS* expression under P_{conG} eliminated the negative feedback necessary for cells to exit competence. Cells possessing this 'feedback bypass' construct become locked in the competent state. Another particularly compelling example is a strain (P_{comG}-*rok*) containing P_{comG}, a promoter expressed only in the competence state, expressing the *rok* protein, a negative regulator of competence (Fig. 8). In contrast to the feedback bypass, the modification strengthens the negative feedback in this case, causing earlier exit from competence. The result is a shorter and less variable distribution of competence residence times, illustrating that a simple change in network topology can dramatically reduce phenotypic variability.

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