1 **Applications of imaging for bacterial systems biology**

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8 Short title: *Applications of imaging for bacterial systems biology*

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Abstract

Imaging has fueled exciting advances in bacterial cell biology, which have led to exquisite understanding of mechanisms of protein localization and cell growth in select cases. Nonetheless, it remains a challenge to connect subcellular dynamics to cellular phenotypes. In this review, I explore synergies between imaging and systems approaches to bacterial physiology. I highlight how single-cell, time-lapse imaging under environmental or chemical perturbations yields insights that complement traditional observations based on population-level growth on long time-scales. Next, I discuss applications of high-throughput fluorescence imaging to dissect genetic pathways and drug targets. Finally, I describe how confocal imaging is illuminating the role of spatial organization in the structure and function of bacterial communities, from biofilms to the intestinal microbiota.

Introduction

Spatiotemporal organization occurs over a wide range of length (from molecular assemblies to multicellular communities) and time (from milliseconds to days) scales in bacteria. To achieve a systems-level understanding of cellular growth, structure, and function, it will be necessary to elucidate how cells establish and coordinate processes over these scales. Direct observation of cell growth and intracellular protein dynamics provides powerful insight into these questions. Every such experiment addresses a system that involves a host of biochemicals: not only do cells often rely on sequential assembly of many proteins to establish subcellular structures, but localization is defined with reference to cell geometry, which in bacteria is determined by the membrane and peptidoglycan cell wall [1]. Moreover, cell shape changes as the cell grows, connecting protein localization to all of the processes essential for proliferation, such as DNA replication, transcription, and translation. Therefore, there is ample potential for synergy in integrating approaches from cell biology and systems biology.

To elucidate genotype-phenotype relationships at the genomic scale, recent chemical genomics studies [2] have exploited high-throughput measures of colony features as a measure of growth. By varying the growth environment with chemical treatments, substrates, and environmental conditions, multidimensional phenotype vectors describing a collection of mutants can be used to study gene essentiality and understand drug action [3]. Moreover, application to genomic-scale libraries empowers clustering of genes involved in a common pathway [3], thereby realizing a systems-level perspective on cellular functions. Nonetheless, a population-level growth metric such as colony size presents only a single perspective on fitness, and will not always accurately

represent the effect of perturbations (be they genetic, chemical, environmental, or other) on bacterial physiology.

There is a growing consensus emerging across biological systems that physical metrics such as cell shape, which by itself provides many phenotypes such as cell width, length, body curvature, and polar morphology, are linked with fitness in meaningful ways. Hence, high-throughput analysis of these metrics across genetic libraries would complement data obtained through chemical genomics. In some bacteria, nutrient-induced increases in growth rate correlate with increased cell volume [4]. Similarly, over the course of a long-term evolution experiment, *Escherichia coli* average cell volume increased approximately two-fold as fitness also increased [5]. Single point mutations in the *E. coli* actin homolog MreB have been found to increase cell size to a similar degree [6,7], with mutations at one particular residue causing a width-dependent increase in competitive fitness during growth; interestingly, this fitness increase was realized only in the presence of certain carbon sources [6], underscoring the importance of probing multiple environments to understand the relationship between cellular physiology and physical features such as cell size. Moreover, population-based measurements often conceal phenotypic heterogeneities in single-cell growth rate and morphology. In the yeast *Saccharomyces cerevisiae*, high-throughput imaging revealed that deletion of many genes increases morphological variation [8]. *Salmonella Typhimurium* persister cells, which represent a small fraction of the population, have been associated with slow growth rates and smaller size [9], indicating that heterogeneities can be critical for a community to survive fluctuating environments or stresses. Thus, cell shape and other single-cell features can provide terminal or time-dependent phenotypes in chemical screens that increase the resolution of our understanding

of gene function.

Fluorescence imaging and multiparametric image analysis can also be used to interrogate cellular processes at the genomic scale, particularly to identify new functions for genes. An imaging-based screen of a *Drosophila* RNA interference (RNAi) library was used to identify >200 genes responsible for mitotic spindle assembly [10]. Key to this study was a high-throughput imaging and quantitative analysis platform that allowed for an unbiased screen across the entire genome, as over half the identified genes were unexpected. Automated analysis of localization dynamics and abundance of fluorescent fusions to ~3,000 *Saccharomyces cerevisiae* proteins was used to reveal fluxes between cellular compartments in response to chemical and genetic perturbations [11]. Unbiased imaging screens may be similarly revelatory in bacteria. Characterization of the localization of fluorescent-protein fusions to most genes in the bacterium *Caulobacter crescentus* revealed that ~10% of the genes have nonuniform localization profiles [12] and uncovered several novel structures including filaments of the metabolic enzyme CTP synthase [13]; this aggregation may be important for efficient channeling of metabolic intermediates [14].

Here, I explore how recent imaging-based initiatives have empowered the investigation of bacterial systems biology across spatial and temporal scales. I also discuss applications of high-throughput fluorescence imaging for dissecting genetic pathways and drug actions.

Imaging can reveal contrasting dynamic phenomena across time scales

Many environmental stresses prompt system-wide responses that affect the rate of cell growth. Thus, assaying changes in growth after hours or days likely conflates many indirect effects and hence may motivate incorrect models of growth regulation. For instance, bacterial growth generally decreases as the osmolarity of the growth medium is increased [15], prompting the hypothesis that turgor pressure directly stimulates growth by driving the mechanical expansion of the cell wall. To distinguish between turgor-mediated effects and indirect, pressure-independent effects of osmolarity changes, a microfluidic flow cell was used to rapidly change osmolarity while quantifying the instantaneous elongation rate via single-cell imaging [16]. On short time scales, although plasmolysis slowed cell elongation, cells nevertheless “stored growth” whereby, upon reestablishment of turgor, they expanded to the length that they would have attained without the osmotic shocks (Fig. 1A) [16]. These experiments reveal a surprising robustness of cell-wall synthesis to turgor fluctuations that is concealed by the decrease in steady-state growth that occurs on longer time-scales [15,16], and highlight the utility of time-lapse imaging of growth responses to perturbations.

Another illustrative example of the power of imaging for unraveling the systems biology of bacteria at various time scales involves the assembly of protein complexes. Although such complexes are thought to facilitate the spatial and temporal coordination of multiple enzymes, it is unclear whether complexes are static or dynamic, and whether either has physiological consequences. Förster resonance energy transfer (FRET) is an imaging-based tool for quantifying the propensity for physical interactions between proteins, and has been employed to map all protein interactions within the *E. coli* chemotaxis pathway [17] including measurements of binding dynamics [18]. Cell-wall synthesis requires the

functions of numerous penicillin binding proteins (PBPs) [1] including PBP2; antibiotic inhibition of PBP2 results in the loss of rod-shaped morphology and eventual cell lysis [19]. Based on the observation that circumferential motion of the *E. coli* actin homolog MreB is dependent on PBP2 activity [20], it was hypothesized that a static complex of PBPs moves with MreB. However, single particle-tracking photoactivated localization microscopy revealed that PBP2 diffuses rapidly [21]. Moreover, cells maintained the same instantaneous growth rate for several generations as PBP2 was depleted (Fig. 1B), suggesting that dynamic association between MreB and PBP2 increases the robustness of growth rate to fluctuations in PBP2 concentration relative to the formation of a stable multienzyme complex [21]. Probing other systems via single-molecule imaging will be critical for determining whether dynamic association is a general paradigm for multienzyme processes, particularly those that globally distribute their activity within the cell.

High-throughput imaging of intracellular localization: interrogating subcellular spatial scales

Given that a sizeable fraction of bacterial proteins [12,22] exhibit localization to specific subcellular locations, high-throughput imaging of localization dynamics holds intriguing prospects for scrutinizing the systems-level establishment of cellular structure and subcellular localization. A recent study used epifluorescence time-lapse imaging to quantify the spatiotemporal dynamics of nearly every *E. coli* protein with a nonuniform localization pattern [23]. This strategy allowed for the quantification of consensus localization patterns across the cell cycle (Fig. 2A), which identified several behaviors that would have been obscured by averaging single-cell fluorescence profiles from snapshots across a

population [23]. These behaviors included several classes of localization patterns of DNA-binding proteins, the relative timing of localization of division-related proteins, and asymmetric protein partitioning after cell division [23], illustrating the effectiveness of fluorescence dynamics as an information-rich phenotypic readout.

Traditionally, investigations of antibiotic mechanisms of action have relied on biochemical assays to determine binding to specific types of molecules (DNA, RNA, protein, etc.) or on the isolation of resistant mutants [24]. However, even if these laborious methods are successful, interactions with other cellular components can alter or obscure the cellular targets. A potent imaging-based methodology is bacterial cytological profiling, wherein changes in morphological features and fluorescence signals are used to cluster antibiotic treatments (Fig. 2B) [25]. For example, subcellular properties such as nucleoid structure display large qualitative differences depending on the antibiotic target, from toroids under treatment with tetracycline (a translation inhibitor) to decondensation with rifampicin (a transcription inhibitor) (Fig. 2C). By exploiting just a few variables such as the localization of membrane, DNA, and permeability stains [25], this method can categorize inhibitors based on five major target pathways to generate hypotheses for the targets of antibiotics with unknown mechanisms of action [25]. In this way, fluorescence imaging can be used similarly to chemical genomics to establish phenotypic profiles, and the combination of these methods could be used in the future to investigate specific pathways.

Spatial organization within bacterial communities: unlocking multicellular spatial scales

185 Confocal imaging makes it possible to correlate molecular processes beyond
186 single cells to the community scale, which is a grand challenge in systems
187 biology. Biofilms are composed of well-defined three-dimensional structures
188 such as wrinkles, concentric rings, and ridges, the formation of which depends
189 on the secretion of extracellular-matrix components [26]. Confocal imaging of
190 slices through an *E. coli* biofilm revealed that these components take on a
191 heterogeneous spatial pattern (Fig. 3A) that is correlated with the differentiation
192 of cells into a two-layer architecture with growing, flagellated cells near the agar
193 surface and a thick layer of matrix-embedded stationary-phase cells adjacent to
194 the air interface (Fig. 3B) [27]. This differentiation is regulated by a collection of
195 molecules including the stationary phase sigma factor RpoS [28] and the biofilm-
196 promoting second messenger cyclic-di-GMP [29], demonstrating that imaging of
197 community architecture provides a window into single-cell gene expression that
198 varies according to the location of each cell in the community.

200 The diverse bacterial community within the gastrointestinal tracts of mammals
201 has generally been investigated via compositional enumeration using sequencing
202 of fecal material [30-35]. However, these population-level measurements ignore
203 the importance of spatial structure in the function of this complex ecosystem,
204 which has multiple length scales spanning micron-sized cells to millimeter-sized
205 tissues. Confocal imaging has provided a high-resolution view of the interactions
206 among bacteria, host cells, and the structure of the local environment.

207 Colonization of some species is maintained by penetration into colonic mucus
208 and crypt channels (Fig. 3C), suggesting that cellular localization within the gut
209 can exert profound effects on microbiota composition [36]. This localization is
210 determined through a molecular conversation with host cells. The antibacterial
211 lectin RegIII γ is essential for maintaining a zone at the surface of the small

212 intestine into which the microbiota do not trespass; RegIII γ regulates both the
213 proximity and identity of bacteria closest to the host epithelium (Fig. 3D) [37].
214 Given the heterogeneity of the intestinal environment, techniques that allow for
215 the imaging of intact gut samples, together with computational tools that can
216 quantify in an unbiased manner features of community organization such as
217 distances among bacterial cells and to host landmarks such as the epithelium,
218 will be required to fully illuminate the structure and function of the microbiota.

Discussion

Looking forward, bacterial cell biology appears poised to capitalize on new imaging techniques, and on advances in computational methods to analyze the imaging data. To complement such developments, genetic advances must be made in tandem. A major challenge for dissecting the function(s) of protein systems has been the major, sometimes catastrophic, phenotypes resulting from the loss of function of any part of these systems. For example, deletion of most of the components of the cell-wall synthesis machinery results in a single rounded morphology [38], obscuring the mechanism responsible for rod-shape determination. Instead, the identification of mutants with subtle changes in cell shape and size [6] will enable quantitative imaging to connect cell morphology, intracellular organization, and physiological phenotypes.

For systems-level investigations, there now exist genomic-scale mutant libraries in several organisms; a combination of chemical genomics and high-throughput imaging of cytological features should provide a unique perspective on the growth and physical phenotypes, respectively, of these mutants. The recent development of dCas9 [39], a mutant allele lacking the Cas9 endonuclease activity used for genome editing in the CRISPR-Cas9 bacterial phage immunity system, introduces the potential for constructing libraries of inducible constructs that repress the transcription of essential genes. Time-lapse imaging of the depletion of essential proteins should provide an unprecedented window into the diversity of cell death phenotypes and synergize with the drug-discovery prospects of cytological profiling.

245 With these genetic tools comes the need for new imaging technologies to capture
246 activity across multiple length and time scales. Compounding the challenge of
247 diffraction-limited resolution for fluorescence imaging in bacteria is the standard
248 epifluorescence approach of capturing two-dimensional projections of a three-
249 dimensional fluorescence distribution. Superresolution techniques provide an
250 avenue for simultaneously breaking the diffraction limit and defining protein
251 localization patterns in three dimensions [40]. Even with high-resolution spatial
252 information about a single protein, its function in the context of other interacting
253 partners may remain enigmatic; FRET should expand applications of
254 superresolution microscopy to the study of the dynamics and localization of
255 protein complexes.

256
257 Advances in modeling will be necessary to capitalize upon new advances in
258 imaging. Even with the knowledge of protein coordinates at high resolution,
259 mapping between the molecular and cellular scales for processes such as cell-
260 wall growth requires the development of computational models that
261 simultaneously capture protein localization, cellular geometry, and the physical
262 properties of cells [41,42]. Such models can then drive tool development for
263 further exploration of system function; for example, successful *in silico*
264 recapitulation of the left-handed macroscopic twisting of the *E. coli* cell body
265 during elongation [43] subsequently led to the development of a novel twisting
266 assay using total internal fluorescence microscopy, which was applied to unravel
267 the phenotypic redundancy of perturbations to MreB and PBP2 [44].

268
269 With the development of high-throughput single-cell imaging, it is now also
270 possible to further extend chemical-genomics data by exploring single-cell
271 phenotypes across genomic libraries under a variety of conditions. Using

principal component analysis to reduce the dimensionality of cell shapes and fluorescence patterns [45,46], it is possible to construct a multidimensional vector based on cytological features [23], yielding imaging-defined phenotypes that complement colony-growth phenotypes of knockouts for gene clustering. Chemical genomics also highlights the conditions most likely to cause growth defects, thereby guiding imaging studies of the effects of these perturbations at a variety of length and time scales using time-lapse imaging. As a further benefit, phenotypic heterogeneity, which has been linked to relevant physiological behaviors such as antibiotic tolerance [47], can easily be interrogated through single-cell imaging.

Although technically challenging, it will be fascinating to evaluate how chemical/genetic perturbations affect the spatial organization of communities and molecules within multicellular communities. For the intestinal microbiota, our capacity for manipulation at genetic, compositional, and spatial levels will require systems approaches and high-throughput imaging and quantitative analysis tools to elucidate the spatial aspects of microbiota function. Similar tools, applied to genomic-scale libraries of pathogens as they have been to host factors using RNAi [48], should also be influential in achieving a more complete understanding of the genetic components of host interactions.

Systems biology promises to ultimately reveal the general regulatory principles present across all kingdoms of life. Imaging is a robust method for probing the effects of environmental perturbations, whether or not the organisms under study are genetically tractable. Ultimately, the prospects for connecting bacterial genotype to phenotype via imaging are brighter than ever.

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Figure Legends

Figure 1: Imaging reveals qualitatively distinct phenotypes at short and long time scales. (A) Using a microfluidic flow cell to alternately apply hyper- and hypoosmotic shocks, imaging-based quantification of cell elongation on short time-scales demonstrated that cells “store” growth during periods of low turgor pressure; despite an apparent decrease in elongation rate, cells reached the length after turgor was restored that they would have achieved if unperturbed [16]. This insensitivity to turgor fluctuations is in contrast to the decrease in growth on longer time-scales at higher osmolarities [15,16]. (B) Using single-particle tracking photoactivated localization microscopy, PBP2 molecules exhibited diffusive motion [21] rather than the directed motion of MreB [20]. This rapid, dynamic association with sites of cell-wall synthesis (inset) enables cells to maintain growth rate for more than two generations as PBP2 is depleted despite the loss of cell shape and eventual death [21].

Figure 2: Applications of high-throughput fluorescence imaging to dissect genetic pathways and antibiotic mechanisms of action. (A) Imaging of a collection of fluorescent-protein fusions to *E. coli* genes revealed a diverse array of localization patterns (modified from [23]). (B) Bacterial cytological profiling, a method that combines imaging data on morphological features and fluorescence patterns of DNA, membrane, and permeability markers with principal component analysis to fingerprint the effects of drug treatment, yields classifications of drugs based on a common mechanism of action (modified from [25]). (C) As an example of the distinct cytological phenotypes, tetracycline treatment results in a toroidal nucleoid while rifampicin decondenses the nucleoid (modified from [25]).

497

498 **Figure 3: Bacterial communities exhibit spatial heterogeneities over large**
499 **length scales that suggest emergent multicellular behaviors.** (A) Confocal
500 imaging of slices of an *E. coli* biofilm (green: thioflavin S fluorescent dye that
501 binds extracellular matrix; overlay with bright-field) shows a structure with a
502 heterogeneous pattern of extracellular-matrix production (modified from [27]).
503 (B) Most matrix is produced by a layer of stationary-phase cells near the air
504 interface, while cells near the nutrient surface continue to grow. (C) Host
505 production of the antibacterial lectin RegIII γ establishes a zone adjacent to the
506 epithelium that prevents the microbiota from encroaching on host cells (modified
507 from [37]). (C) Localization of *Bacteroides fragilis* cells to the colonic crypts is
508 connected with stable intestinal colonization (modified from [36]).