Building spatial synthetic biology with compartments, scaffolds, and communities

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Abstract

Traditional views of synthetic biology often treat the cell as an unstructured container in which biological reactions proceed uniformly. In reality, the organization of biological molecules has profound effects on cellular function: not only metabolic, but also physical and mechanical. Here we discuss a variety of perturbations available to biologists in controlling protein, nucleotide, and membrane localization. These range from simple tags, fusions, and scaffolds to heterologous expression of compartments and other structures that confer unique physical properties to cells. Next, we relate these principles to those guiding the spatial environments outside of cells, such as the extracellular matrix. Finally, we discuss new directions in building intercellular organizations to create novel symbioses.

Introduction

Spatial organization of biological molecules is foundational to life. The localization of proteins to distinct regions of the cell – be they as simple as the cytosol, membrane, and nucleoid – restricts and directs enzymatic interactions. The division of cellular contents into compartments further separates otherwise incompatible chemical and enzymatic microenvironments. And finally, the organization of proteins into polymeric structures endows cells with the ability to control their mechanical properties and exert forces in their environments.

Synthetic biology is typically understood to mean endowing cells with novel functions based on genetic manipulations. Of course, modifying transcription necessarily modifies the protein environment in the cell as well. However, simply expressing proteins that are assumed to act independently of their environment severely limits the potential of this discipline to generate new cellular behaviors. Here we describe a range of engineered modifications to cellular organization. Some of these have reached mainstream use in studying and perturbing cellular functions, while others have unrealized potential.

Directing protein localization

A fundamental source of enzymatic organization within cells is the direction of proteins to specific compartments or locations within the cell (Blobel and Sabatini 1971). These can include the nucleus (Silver et al. 1984), mitochondria (Rapaport 2003), chloroplasts (Tian and Okita 2014), vacuoles (Washida et al. 2009), or, in the case of gram-negative bacteria, the periplasm (Danese and Silhavy 1998). As the responsible elements are N-terminal sequences on the proteins, this localization mechanism can be readily exploited to direct proteins to novel locations. For example, this technology has recently been combined with variable

splicing to create reporters with differential localization to plant cell chloroplasts, peroxisomes, and the cytosol (Voges et al. 2013).

In addition to directing proteins to specific localizations in the cell, their oligomerization state can be modulated. Glutathione S-transferase (GST) tags typically used for affinity purification form dimers, (Parker et al. 1990) and this property alters protein function, such as by increasing the binding affinity of a DNA-binding protein (Niedziela-Majka et al. 1998). This property has been exploited as a test of the functional role of oligomeric state (Lee et al. 2003).

While induced hetero- or homo-dimerization is a common technique in molecular and cell biology, these tools and approaches have nevertheless been expanded in interesting ways. In addition to localizing individual proteins, they have been used to move whole organelles: a synthetic heterodimerization “crutch” linking the ER to mitochondria was used in a screen to identify factors that naturally co-localize these organelles (Kornmann et al. 2009). Dimerization can also be inducible as well as constitutive: the FK506-binding protein (FKBP) can be used as a tag that dimerizes upon withdrawal of its chemical ligand; mutants can reversibly convert between monomers and dimers (Rollins et al. 2000). Futhermore, engineered phytochrome-PIF interaction pairs are induced to dimerize with light; this permits optogenetic control of protein spatial localization, even in regions of single cells (Levskaya et al. 2009). And finally, dimerizing tags are available in a variety of affinities, including those that form covalent bonds: the SpyCatcher and SpyTag system is a derivative of a bacterial fibronectin-binding protein that irreversibly forms an isopeptide bond upon interaction (Zakeri et al. 2012).

In addition to directing the localization of single proteins, tags can be used to make larger aggregates, which can in turn be used to probe cellular function. For example, FM repeats (tandem repeats of FKBP domains) have been fused to enzymes in the Golgi apparatus, causing them to polymerize into aggregates upon withdrawal of the ligand. The accumulation of these polymerized enzymes in the cis or trans Golgi have been used to investigate models of cisternal maturation (Rizzo et al. 2013; Rivera et al. 2000; Lavieu et al. 2013).

Scaffolds

Directing the localization of proteins can be used to not only probe cellular function, but also enhance enzymatic activity or to achieve a metabolic or signaling effect. Multiple proteins can be scaffolded, or coordinated with specified geometry, to enhance their activity. The exact nature of enzymatic enhancement on scaffolds is likely to be a combination of several factors including a high local concentration of substrates and their “channeling” within a shared hydration shell. The latter has been proposed after introducing an inert bridge protein between glucose oxidase and horseradish peroxidase on a DNA scaffold *in vitro*, thereby increasing the enzymatic output compared to similar scaffolds lacking the bridge (Fu et al. 2012).

Scaffolding to enhance enzymatic activity occurs naturally in cells. For example, fatty acid synthetase is a single protein with seven enzymatic centers; their direct translational fusion is likely to confer metabolic benefits (Smith 1994). Multiple polypeptides can also be brought together by protein-protein interactions: in *B. subtilis*, the nonribosomal peptide synthetases and polyketide synthetases (NRPS/PKS) enzymes assemble to form a ~2.5 megadalaton complex; these complexes are further localized together in one focus in the cell (Straight et al. 2007).

From a synthetic biology perspective, the simplest engineered enzymatic enhancement is by direct fusion of one protein to another. This approach was used to achieve 15-fold enhancement of resveratrol production in yeast and human cells (Zhang et al. 2006b). Scaffolds, however, offer the additional flexibility of linking components by protein-protein interactions. An early example is a modification of the bacterial cellulosome, which is an assembly that organizes cellulose-degrading enzymes. This work enhanced the natural cellulolytic

activity of *Clostridium* by combining enzymes and binding domains from different species (Fierobe et al. 2001). A synthetic protein scaffold with more distantly related components was created in 2009 by recruiting yeast metabolic enzymes to metazoan proteins that naturally interact to function in signaling. This improved melvalonate production between 3 and >70-fold in *E. coli*, depending on the concentration of enzymes expressed – at lower concentrations of enzymes, scaffolds were more beneficial, presumably because of a greater relative increase in effective concentration (Dueber et al. 2009). These protein scaffolds are limited in the potential spatial organization of the interacting domains, and by the fact that their assembly is not by nature polymeric.

By contrast, the modular properties of nucleic acids can be used to create structures that offer both theoretically infinite size and fine control of enzymatic orientation. RNA can be constructed scaffolds in varying dimensionalities – 0, 1, and 2D scaffolds can be constructed by varying the base pairing between modular segments (Delebecque et al. 2012). The number of interacting proteins is limited only by the number of aptamers (and aptamer binding proteins fused to the enzymes of interest). Much like protein scaffolds, RNA scaffold can achieve >50-fold enzymatic enhancements in *E. coli*, though the limited stability of RNA may affect scaffold performance over long time scales (Delebecque et al. 2012).

The potential problems with RNA stability may be alleviated with the use of DNA scaffolds instead, though this platform presents its own inherent limitations. For example, DNA topology is limited to a single dimension, and supercoiling is also a potential problem. Nevertheless, DNA scaffolds have been used to generate up to 5-fold enhancements in resveratrol, 1,2-propanediol, and mevalonate production in *E. coli* (Conrado et al. 2012). Each enzyme was fused to a zinc-finger protein; the diversity of this protein family suggests that many enzymes could be arranged in a specific array.

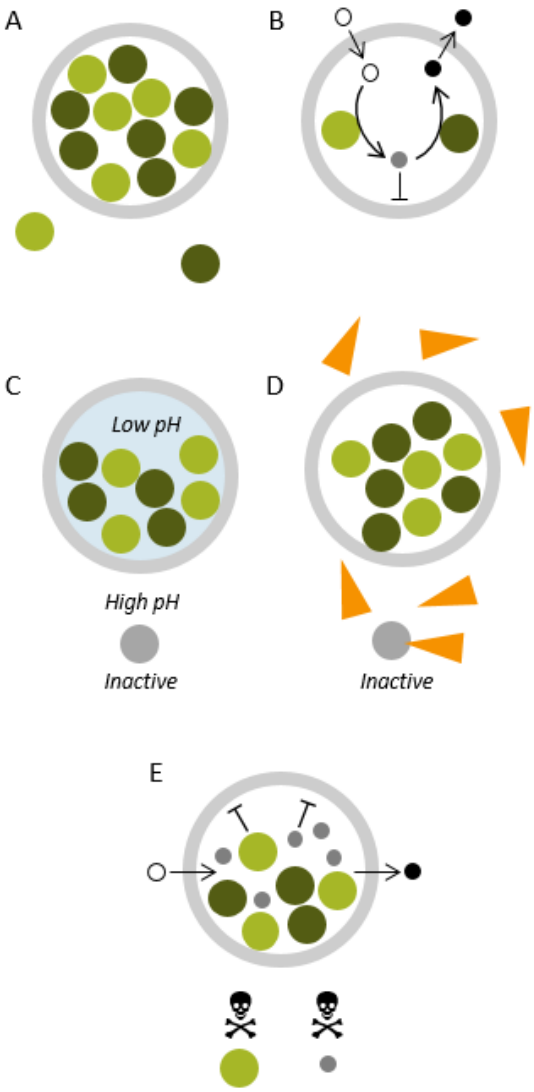

Figure 1. Potential benefits of compartmentalization (with gray rings representing intracellular compartments). A) Concentration of enzymes. B) Increased concentration of intermediates through a selectively permeable diffusion barrier. C) Maintenance of a chemical microenvironment essential for enzymatic function. D) Protection of enzymes from molecules from deactivators or competitors. E) Isolation of toxic enzymes of intermediates from the rest of the cell.

Membrane barriers

Though scaffolds may organize high concentrations of enzymes in specific orientations, the surrounding environment remains essentially identical to the cellular compartment in which they form. This could be disadvantageous for several reasons (figure 1). First, reactions may produce toxic products, byproducts, or intermediates, such as the formaldehyde formed as an intermediate of methanol metabolism (Fassel et al. 1992). Second, enzymes may need to be insulated from certain substrates to prevent non-productive side-reactions – for example, RuBisCO can catabolize a reaction with molecular oxygen rather than carbon dioxide, drastically reducing enzymatic efficiency. Third, enzymes may require a specific chemical

environment in terms of pH, redox potential, or ionic strength that is distinct from the rest of the cytosol, such as is created in mitochondria.

Since most compartments in eukaryotes are membrane-based, it is no surprise that many engineering attempts have focused on membrane encapsulation. The most readily implemented form of this technology is the use of targeting sequences to localize enzymes to existing organelles. For example, up to five enzymes in an isobutanol synthesis pathway have been directed to yeast mitochondria; there, they provide modest (~3-fold) enhancements over expression in the cytoplasm (Avalos et al. 2013).

In addition, new membrane topologies can also be created from the ER, often through the overexpression of foreign membrane-associated proteins. In tobacco plants, viral proteins form whorled membrane structures termed z-membranes that appear to derive from the endoplasmic reticulum (Gong et al. 1996). By making fusion to these viral proteins, various enzymes can be localized on the cytoplasmic surface of these membranes. In mammalian tissue culture cells, ER derivatives having a cuboid or crystalline morphology (termed organized smooth ER, or OSER) can be generated by expression of certain proteins (Yamamoto et al. 1996; Snapp et al. 2003). This morphology can be produced with chimeric proteins that inducibly dimerize, enabling the topology to be dynamically controlled (Lingwood et al. 2009).

Vesicles have also been generated from other membrane sources; for example from the cell membrane of *E. coli* (Figure 2). This was accomplished by overexpressing foreign bacterial glucosyltransferases (Eriksson et al. 2009) or by expressing eukaryotic calveolin (Walser et al. 2012). The latter strategy produces morphologically normal calveolae 50nm in diameter that encapsulate contents derived from the periplasm. Furthermore, by making fusions to the calveolin, the vesicles can be functionalized with proteins that allow them to be targeted to specific cell types after purification.

In addition to directing encapsulation within membrane-bound compartments, we are also able to control release of contents from membrane-bound compartments, such as the cell itself. This takes the shape of inducible bacterial lysis, reviewed in (Rice and Bayles 2008). These strategies can involve the expression of lysozyme and pore-forming proteins in response to varied triggers (Pasotti et al. 2011). However, bacteria can also be made to kill specific foreign strains of bacteria with the use of specialized protein machines. For example, pyocins are contractile apparati resembling phage tails that kill target bacteria by depolarizing bacterial membranes (Uratani and Hoshino 1984). Pyocins can be engineered to kill varied bacteria by substitution of their tail fiber proteins from strain-specific phage (Williams et al. 2008). R bodies, proteinaceous toxin delivery devices resembling needles that can extend up to 20um long, function to mix the cytoplasms of the expressing bacteria with that of a predatory eukaryote in response to the low-pH environment of a phagosome (reviewed in (Pond et al. 1989)). As R bodies can break foreign membranes to release encapsulated protein cargo (Polka and Silver 2016), they could be used to release or mix contents from multiple cells, synthetic vesicles, or compartments.

Microcompartments

Though further afield from the traditional conception of eukaryotic organelles, proteinaceous compartments may offer a more flexible, robust, and practical platform for engineering compartmentalization. Perhaps the most versatile are microcompartments: icosahedral protein structures, typically between 100-200nm in diameter, found in diverse bacteria. They naturally encapsulate and insulate enzymes from the cellular environment with faceted, selectively-permeable shells composed of pentameric and hexameric proteins. Three of the best studied microcompartment classes include Pdu (propanediol utilization) microcompartments; Eut (ethanolamine utilization) microcompartments; and carboxysomes, which contain carbonic anhydrase and

RuBisCO. Carboxysomes form two major families: alpha and beta carboxysomes, which differ in the type of RuBisCO incorporated and also by the importance of the matrix in organizing the shell (Rae et al. 2013; Schmid et al. 2006). Microcompartments are now known to be more widespread than originally thought, with 17% of sequenced genomes bearing sequences thought to encode microcompartments of these and other types (Jorda et al. 2013).

Microcompartments are of particular interest because they can be assembled from a small number of protein components, and are therefore more portable to heterologous expression systems. For example, Pdu compartments from *Citrobacter freundii* have been made in *E. coli* by heterologous expression (Parsons et al. 2008); the empty shell can also be made by expressing only five proteins (Parsons et al. 2010). Because the lumen components were not transferred in the latter study, the compartments are hollow, leaving the opportunity for them to be filled with alternate cargo, as discussed below. Pdu compartments from *Salmonella entericum* have also been made in *E. coli* (Sargent et al. 2013), and empty Eut shells have been reported to form in *E. coli* as well (Choudhary et al. 2012). Finally, alpha carboxysomes from *H. neapolitanis* have been expressed in *E. coli* from a single operon (Bonacci et al. 2012). Complete beta carboxysomes have so far resisted heterologous expression, perhaps because of a more complicated genetic organization spanning several operons. However, expression of beta carboxysome shell proteins in *E. coli* does produce capsid-like structures (albeit smaller than carboxysomes) which associate with heterologous cargoes tagged with targeting sequences (Cai et al. 2016).

Heterologous expression of carboxysomes, even with their native contents, is of particular interest in light of the goal of improving carbon fixation in plants. Carbon fixation is a metabolic bottleneck for many plants, and the introduction of carboxysomes into the cells of agriculturally important organisms could theoretically increase crop yields by 36-60% (McGrath and Long 2014). Though the cyanobacterial RuBisCO is faster than plant RuBisCO, it is also more sensitive to molecular oxygen, and thus is detrimental to the growth of its host without encapsulation. Thus, encapsulating this enzyme will be necessary to reap any metabolic benefits of cyanobacterial RuBisCO. Progress has been made toward this end in the expression of carboxysome components in tobacco, with shell and RuBisCO both being successfully expressed (Lin et al. 2014b, 2014a). However, a complete carboxysome in plants has yet to be achieved.

For microcompartments to have novel functions, heterologous cargoes must be targeted to them. In general, this is accomplished by exploiting natural mechanisms of targeting cargos to the microcompartment lumen. Across many families of micrcompartments, short regions at the termini of cargo proteins are thought to form small alpha helices that bind to the interior surfaces of shell proteins (Fan et al. 2012). The best-developed platform for heterologous targeting is the Pdu microcompartment system. The terminus of PduP from *Salmonella enterica* is necessary and sufficient to drive cargos to the microcompartment. GFP tagged with Pdu^{1-18} can not only localize to the compartments with fluorescence microscopy, but it is also protected from immunoprecipitation from cell lysates after disruption; this suggests it is located inside the compartment rather than simply associated with the exterior. In addition to PduP, a second enzyme, PduD, also contains an 18 amino acid sequence sufficient for targeting heterologous cargoes to the *S. enterica* Pdu compartment. PduD is also required for recruiting the other two members of its natural complex, PduCDE, suggesting that not only direct fusions, but also protein-protein interactions, could be used to localize enzymes to the *S. enterica* compartment. (Fan and Bobik 2011).

Targeting of engineered cargoes should ideally be functional in a heterologous system. PduC, D and V can all direct GFP to colocalize with heterologously expressed Pdu compartments in *E. coli* (Parsons et al. 2010). Also in this system, $PduP^{1-18}$ and $PduD^{1-18}$ can be used to target two enzymes involved in ethanol production to the compartment, thereby increasing ethanol yield (Lawrence et al. 2014). It is possible that the enzymes are located on the outside, rather than the inside of the compartment, but nonetheless the Pdu structure succeeds

as a scaffold. Finally, a targeting system exists in beta carboxysomes: the C terminus of CcmN is responsible for directing it to the *S. elongatus* carboxysome (Kinney et al. 2012). The peptide is also necessary for the formation of the beta carboxysome shell, though it does not exist in alpha carboxysomes. While the CcmN peptide has not yet been shown to localize heterologous cargo, proteins can be targeted to the lumen of the carboxysome by fusion to full-length, native cargos, like RuBisCO. These fusions, at least in the case of fluorescent proteins, do not impact carboxysome structure, function (Savage et al. 2010), or the establishment of an internal oxidizing microenvironment (Chen et al. 2013), implying that carboxysomes could accommodate small enzymatic cargoes and retain their function.

In order to optimize enzymatic activity in any engineered compartment, the chemical microenvironments may need to be engineered. The concentration of small molecules inside microcompartments is thought to be controlled by the size and charge of the pores formed in the middle of shell protein hexamers; by exchanging residues in the pores, the selectivity of the permeability barrier could theoretically be changed. As a step toward this goal, chimeric carboxysome shells have been made. In these experiments, a shell protein (CsoS1) from *Prochlorococcus marinus* colocalized with *S. elongatus* carboxysomes, and even structurally complemented a deletion of their major shell protein (Cai et al. 2014). Though CsoS1 shares less than 50% similarity with *S. elongatus* shell proteins, this study shows that components of alpha (*P. marinus*) and beta (*S. elongatus*) carboxysomes can be interchangeable, expanding future engineering possibilities.

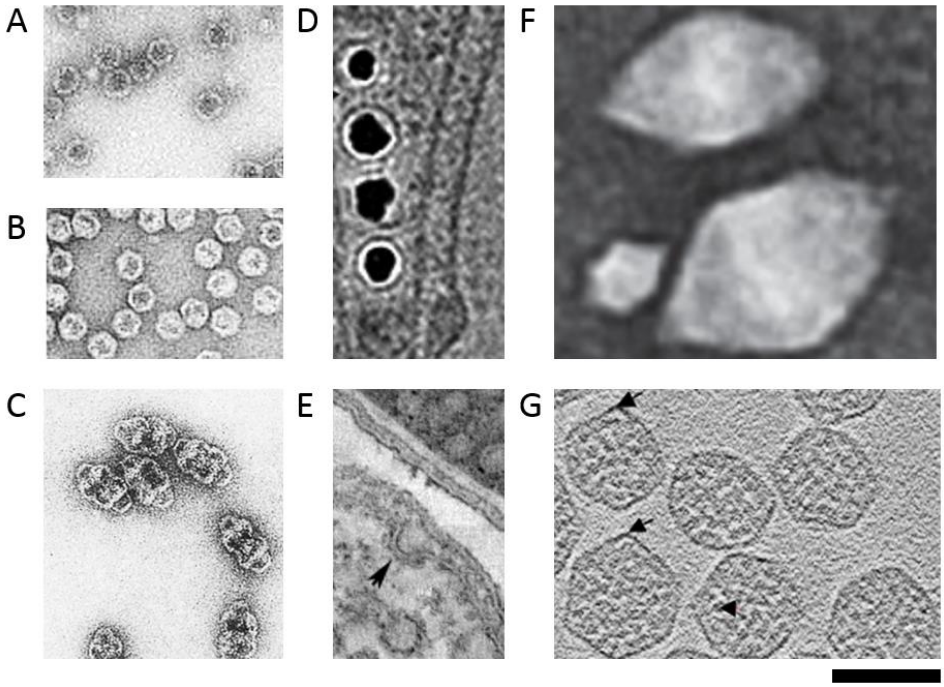

Figure 2. Transmission electron micrographs of cellular compartments, scaled to their relative size. Scale bar: 100μm. A) Lumazine synthase (Zhang et al 2006). B) Encapsulin (Sutter et al 2008). C) Vaults (Kedersha et al 1986). D) Magnetosomes near the cell membrane (Komeili et al 2006). E) Calveolae; budding from the cell membrane indicated with an arrow (Walser et al 2012). F) Gas vesicles (Pfeifer 2012). G) Alpha carboxysomes; arrows indicate vertexes, triangle indicates RuBisCO (Schmid et al 2006).

In addition to the permeability of the shell, the functionality of an engineered microcompartment would be influenced by its size: not only through the number of molecules that could be encapsulated, but also in the surface to volume ratio, which may influence the compartment's microenvironment. There is a large natural range in microcompartment diameters – from 40nm *Haliangium ochraceum* compartments to 500nm beta carboxysomes in *Spirulina platensis* (Lassila et al. 2014; Rae et al. 2013). In addition, the size of at least one species of beta carboxysome can be modulated genetically. In *S. elongatus*, the protein CcmM is localized to the lumen of carboxysome, where it organizes RuBisCO into a solid matrix. CcmM is present in two isoforms; deleting the short isoform drives the formation of small carboxysomes (Long et al. 2010), while fusing a tag to CcmM drives the formation of larger carboxysomes (Long et al. 2007).

Encapsulins

While microcompartments have shells made up of multiple proteins, a second type of bacteria compartment, encapsulins, have only one shell protein and are characterized by smaller diameters, approximately 30nm. They typically encapsulate proteins involved in oxidative stress responses, such as ferritin-like proteins or peroxidases.

The first encapsulin was discovered in *Pyrococcus furiosus*. This compartment is made out of only one protein which functions both as its shell and enzymatic cargo (Namba et al. 2005). Related compartments were later found in other organisms, including *Thermatoga maritima;* this encapsulin is composed of 60 monomers of a single subunit that form a cage 24nm in diameter (Sutter et al. 2008). The crystal structure of the shell protein shows structural similarity to viral capsids, but like microcompartments, there is no homology on the sequence level. These authors also expressed the two-component encapsulin operon from *Brevibacterium linens* in *E. coli* and found that the cargo, a single oligomeric complex, is localized within the lumen of the compartments, supporting the hypothesis that a C-terminal sequence targets cargos to encapsulin lumens.

The purified encapsulin from *Rhodococcus jostii* can be assembled and disassembled in vitro in a pH-dependent fashion, and the assembly of the encapsulin enhances the enzymatic activity of the peroxidase cargo (Rahmanpour and Bugg 2013). The *Rhodococcus erythropolis* encapsulin is capable of both being expressed heterologously in *E. coli* and of packaging heterologous cargo: protein tagged with the C-terminal 37 amino acids of its natural cargo is protected from trypsin proteolysis in the presence of the shell (Tamura et al. 2015).

Myxococcus xanthus also produces an encapsulin compartment; the native compartment co-purifies with not one, but three cargo proteins. This suggest that these structures could enclose multiple proteins and perhaps support encapsulation of whole enzymatic pathways (McHugh et al. 2014). This encapsulin can be expressed in *E. coli*, although the heterologously expressed protein forms some 18nm compartments as well as those of the native 32nm size. Furthermore, encapsulins are also being explored as a platform for targeted drug delivery (Moon et al. 2014).

Lumazine synthase

A third type of proteinaceous compartment found in bacteria is composed of lumazine synthase, an enzyme involved in riboflavin biosynthesis. In some bacteria, yeast, and plants, this protein forms pentamers (Baldi et al. 2000; Meining et al. 2000), but in other species these pentamers are further assembled into capsids – namely in *Bacillus subtilis* (Ladenstein et al. 1994) and *Aquifex aeolicus* (Zhang et al. 2001). The capsids from the latter system, termed AaLS, can be expressed and purified from *E. coli*; they assemble, in vitro, into compartments either 16 or 30nm, depending on pH and buffer conditions. (Zhang et al. 2006a) They have no native cargo.

To facilitate encapsulation of heterologous cargoes, the internal surface of lumazine synthase has been mutated to increase electrostatic interactions by charge complementarity. This strategy has been useful for encapsulating proteins bearing a positively-charged stretch of 10 arginine residues on the C-terminus co-expressed with these modified AaLS compartments in *E. coli* (Seebeck et al. 2006). Furthermore, this approach can raise the tolerated expression level of HIV protease, which is toxic to *E. coli* (Wörsdörfer et al. 2011b). This selection formed the basis of a screen for mutants of AaLS that could better protect its host, presumably through improving encapsulation owing to increased negative charges on its interior surface. These improved capsids, called AaLS-13, can be isolated and loaded *in vitro* with monomers of GFP that have

been mutated to bear 29 additional positively-charged residues (Wörsdörfer et al. 2011a) or even with similarly engineered human ferritin (Beck et al. 2014). Ferritin is itself a small microcompartment, approximately 12nm in diameter that binds and stores iron. Thus, the encapsulation of human ferritin into *A. aeolicus* lumazine synthase generates a nested synthetic organization. It is also reminiscent of natural encapsulins that can bear ferritin cargoes, but here the interaction is dependent on engineered properties of the compartments, which come from different domains of life.

Lumazine synthase capsids have been engineered to produce vaccines because they can act scaffolds for antigen presentation and are themselves immunogenic (Alfano et al. 2015; Du and Wang 2015). They have also been fused to a chelating agent for use as a contrasting agent for MRI (Song et al. 2015).

Vaults

Not all protein compartments have a bacterial origin. Vaults, lemon-shaped barrels 70nm long and 40nm in diameter, are found in the cytoplasm of eukaryotic cells, but their function is unclear. They are naturally composed of three proteins and an untranslated RNA (Kickhoefer et al. 1993; Kedersha and Rome 1986), but can be purified from insect cells by expression of the major structural protein alone (Stephen et al. 2001). A domain called INT, found on one of the non-essential vault proteins (Kickhoefer et al. 1999), is sufficient to target heterologous cargoes to vaults expressed in insect cells (Kickhoefer et al. 2005). These INT-tagged proteins can be visualized in cryoEM reconstructions as two rings of additional density encircling each half of the barrel. INT tagging also has biochemical effects: targeting a fluorescent protein with vaults with INT reduces the speed with which fluorescence decreases in response to high ionic strength, suggesting that they are partially protected from solution. Furthermore, INT-tagged luciferase is functional, yet displays reduced kinetics, suggesting that enzymes can function inside vaults (Kickhoefer et al. 2005). From an engineering perspective, this approach has been used to package a *Chlamydia trachomatis* protein into vaults for vaccine development. (Zhu et al. 2014). Manganese peroxidase packaged within vaults is more stable, suggesting potential benefits for remediation of pollutants(Wang et al. 2015).

Since vaults are dynamic structures, cargoes can be loaded in vitro (Poderycki et al. 2006); the two halves of the vault can even completely exchange (Yang et al. 2010). These approaches can be used to package inorganic compounds, like Ni-NTA-nanogold, into the vaults (Goldsmith et al. 2009).

Vaults proteins can also be engineered to gain new functions. For example, an amphipathic helix was fused to the N terminus of the major vault protein, causing lipids to be encapsulated in the vault (Buehler et al. 2014). The lipid-containing vaults bind with greater avidity to fat-soluble drugs. Purified vaults are readily taken up by cells (Kickhoefer et al. 2005), but fusing a viral endosomolytic peptide enhances the delivery of DNA and other molecules to the cytoplasm (Han et al. 2011).

Magnetosomes

In nature, the function of complex structures is not limited to isolating enzymatic activity. Packing of specialized materials - metals and gas - can serve important roles in natural cellular migration, and have been exploited for diverse practical purposes as well.

Magnetosomes are intracellular structures that enable some bacteria to position themselves using the earth’s magnetic field. Morphologically, they are invaginations of the cell membrane that enclose magnetite crystals 50nm in diameter; these are spatially distributed through the cell by an actin homolog. (Komeili et al. 2006) Though they are encoded by large islands of genes, magnetosomes from *Magnetospirillum gryphiswaldense*

have been heterologously expressed in *Rhodospirillum rubrum,* with their order and morphology preserved (Kolinko et al. 2014).

MamC, a protein that localizes to the membrane, has been tagged with cameloid antibody fragments (ie "nanobodies") in *M. gryphiswaldense.* Since this fusion enables interactions between magenetosomes and the nanobody target protein both *in vitro* and *in vivo*, it can be used to generate an intracellular "nanotrap" that localizes natural proteins to the magnetosome in living cells (Pollithy et al. 2011).

Magnetosomes also have many possible applications after purification, for example, their patterning on a surface could be used to create a magnetic memory device (Galloway et al. 2012). Furthermore, a phosphohydrolase has also been fused to MamC in *Magnetospirillum magneticum* to create magnetosomes that could be purified and used *in vitro* to degrade a pesticide (Ginet et al. 2011). Magnetosomes from this organism can also be functionalized with multiple enzymes with defined stoichiometry for cellulose hydrolysis, among many other fusion partners (Honda et al. 2015).

Magnetic particles in yeast have also been produced, though these inclusions lack the structure of magnetosomes (Nishida and Silver 2012). The particles will form naturally in *S. cerevisiae* in the presence of ferric citrate, but the yield and size of the particles can be increased by knocking out a yeast iron transporter and heterologously expressing human ferritin.

Gas vesicles

Gas vesicles constitute another type of genetically encoded, specialized compartment. Used to regulate bacterial buoyancy during migration to appropriate parts of the water column, gas vesicles are protein-enclosed structures that exclude water and so contain only gas. They have a variety of physical morphologies, including spindles, cylinders, and bicones, and they can grow to sizes ranging from 100nm to 2μm long, depending on the species. (Pfeifer 2012)

Bacillus megaterium gas vesicles have been heterologously expressed in *E. coli* (Li and Cannon 1998). Because gas vesicles make cells float, they have been proposed as a tool to facilitate harvest of bacteria in an industrial setting through the formation of a pellicle. Purified gas vesicles can also be used to display antigenic peptides (Stuart et al. 2001), to oxygenate tissue culture media (Sundararajan and Ju 2006) and to serve as contrast agents for ultrasound imaging (Shapiro et al. 2014).

Cell morphology and the cytoskeleton

In eukaryotic cells, long-range order is achieved through dynamic cytoskeletal proteins and reaction-diffusion systems. If these systems could be controlled, cell shape, migration, and cell size could all be potentially regulated. Currently, the eukaryotic actin and microtubule cytoskeletons can be exploited to direct cargos to specific positions within cells (reviewed in (Goodman et al. 2012)). For example, a DNA binding domain fused to a portion of a microtubule motor, a dynein light chain, can enhance transfection affinity, presumably because the reagent enables the microtubule cytoskeleton to move the DNA toward the nucleus (Toledo et al. 2012). Furthermore, targeting peroxisomes to motors can direct these organelles toward either the nucleus or the periphery of the cell, depending on the polarity of the motors (Kapitein et al. 2010a, 2010b).

While bacteria and archaea lack ubiquitous, persistent load-bearing cytoskeletons in the traditional sense, they do have protein polymers and reaction-diffusion systems that regulate division, cell wall deposition, and DNA segregation. In addition, these proteins also control the position of compartments like carboxysomes (Savage

et al. 2010) and magnetosomes (Komeili et al. 2006). Therefore, these proteins could play an important role in specifying the intracellular location of synthetic structures in the future.

The extracellular environment

Biological organization does not stop at the plasma membrane. In bacteria, the extracellular environment provides a means for protection against toxins and mechanical forces. This matrix can also be modified for scaffolding applications and to create novel structural and mechanical properties. For example, curli protein forms amyloid fibrils and is a major component of the extracellular matrix made by *E. coli* and related bacteria. By placing curli protein under an inducible promoter, biofilm production can be induced with an external signal (Chen et al. 2014). Curli fibrils have also been tagged with metal binding peptides to enable them to bind gold nanoparticles and quantum dots to make “nanowires,” and the proportion of tagged curli subunits can be regulated temporally with different inducers. (Chen et al. 2014) Additionally, curli firbrils can be covalently decorated, via SpyCatcher/SpyTag, with any protein of interest (Nguyen et al. 2014).

The extracellular matrix of eukaryotic cells can also be modified to influence behavior. For example, a SpyCatcher/SpyTag-crosslinked elastin network that incorporates Leukemia Inhibitory Factor has been used as a growth medium for mouse embryonic stem cells; this medium maintains stem cell pleuripotency (Sun et al. 2014). In theory, this matrix could be genetically encoded.

In addition to matrixes, bacteria make other extracellular structures with biological relevance that are amenable to engineering. For example, *Geobacter sulfurreducens* makes extracellular magnetite particles, the size and composition of which can be altered by changing the composition of available metals (Byrne et al. 2013). A second example are exosomes, extracellular vesicles up to 200nm in diameter which naturally package mRNA and proteins. Exosomes are used to communicate between cells, and have been engineered to incorporate heterologous cargoes (Marcus and Leonard 2013). Similarly, in gram-negative bacteria, outer membrane vesicles (OMVs) are produced naturally for many purposes, including carrying toxins and protecting bacteria from antibiotics and phage (reviewed in (Kulp and Kuehn 2010; Avila-Calderón et al. 2015)). They are also the focus of active engineering efforts, for example in the production of vaccines (Chen et al. 2010).

Symbiosis

Finally, compartments within cells can be cells themselves. For example, diatoms such as *Hemiaulus hauckii* internalize cyanobacteria like *Richelia intracellularis;* these contain dedicated nitrogen-fixing cells called heterocysts. The specialized compartments allow the cyanobacteria, and thus the diatom, to reap the benefits of both nitrogen fixation and oxygenic photosynthesis (Carpenter et al. 1999; Schouten et al. 2013).

Recapitulating endosymbiotic interactions is a goal of particular interest because it could lead to the development of synthetic organelles. For example, cyanobacteria have been engineered to escape from lysosomes; they have been shown to survive inside zebrafish and mammalian macrophages (Agapakis et al. 2011). Notably, similarly treated *E. coli* were severely cytotoxic in these experiments, suggesting that *S. elongatus* may be inherently better suited to engineering stable endosymbiotic interactions in the future.

Cells do not need to engulf – or be engulfed by – their symbiont in order to take advantage of inherently dissociated cellular components and capacities. Organization into a consortium allows for the division of labor among different cells, which effectively act as individual compartments. In the example of *Chlorochromatium aggregatum*, the consortium is made up of a single betaproteobacterium surrounded by green sulfur bacteria (Overmann 2010). Though the non-motile green sulfur bacteria can live alone, they benefit from the specialized motility of the flagellated betaproteobacterium that exhibits chemotaxis towards light, despite the bacterium not

being photosynthetic (Frostl and Overmann 1998). The motility acquired through symbiosis gives the green sulfur bacteria an advantage over other non-motile competition. Presumably the betaproteobacteria also benefits through the provision of photosynthetically fixed carbon.

Often this division of labor endows consortia with capacities far beyond those of individual species. Visually stunning examples of this are microbial mats where colored striations represent different metabolic capacities: gold, green, pink, peach, or dim green layers correspond to organisms growing together capitalizing on different photosynthetic machinery with metabolisms that differ in ability to withstand exposure to oxygen, reduce sulfate, and fix nitrgoen (Stal et al. 1985).

Symbiotic consortia appeal to engineers for two reasons: 1) division of labor lends itself well to modularity, and 2) it is widely believed that consortia are more stable than monocultures. In this vein, synthetic biologists have engineered consortia made up of different strains of the same organism as well as symbioses involving multiple species. There have been many applied examples of the utility of symbiosis, especially in isobutanol and lipid production from sunlight or cellulosic feedstocks (Minty et al. 2013; Ortiz-Marquez et al. 2012; Do Nascimento et al. 2013). While at their simplest these artificial consortia are merely novel combinations of existing wild-type strains, there are many engineering interventions that can improve their stability. For example, engineered auxotrophies force interdependence (Müller et al. 2014; Shou et al. 2007; Wintermute and Silver 2010), and quorum sensing systems can be used to facilitate communication between consortium members(Brenner et al. 2007; Weber et al. 2007; Chen et al. 2015).

It is worth noting that symbiosis can also involve metazoan partners. Consider the bioluminescence provided by *Vibrio fischeri* in the bobtail squid (Naughton and Mandel 2012), amino acids provided by bacterial endosymbionts in aphids (Hansen and Moran 2011), organic carbon and oxygen provided by photosynthetic algae in corals (Roth 2014), and our own microbiomes. These and other examples of natural symbiosis provide will provide fertile ground for future engineering efforts.

Concluding remarks

The ultimate goal of these engineering efforts is to control not only the contents of cells, but also the spatial relationship of these contents to one another. Given that the activity of proteins is dependent on both their potential interacting partners and their local chemical environment, cellular organization must be manipulated to explore the full range of potential biological activity. In order to gain this mastery, synthetic biologists must adopt into their toolkit some microscopy and biochemical methods associated with traditional cell biology.

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Figure Legends

Figure 1. Potential benefits of compartmentalization (with gray circles representing intracellular compartments). A) Concentration of enzymes. B) Increased concentration of intermediates through a selectively permeable diffusion barrier. C) Maintenance of a chemical microenvironment essential for enzymatic function. D) Protection of enzymes from molecules from deactivators or competitors. E) Isolation of toxic enzymes of intermediates from the cell.

Figure 2. Transmission electron micrographs of cellular compartments, scaled to their relative size. Scale bar: 100μm. A) Lumazine synthase (Zhang et al 2006). B) Encapsulin (Sutter et al 2008). C) Vaults (Kedersha et al 1986). D) Magnetosomes near the cell membrane (Komeili et al 2006). E) Calveolae; budding from the cell membrane indicated with an arrow (Walser et al 2012). F) Gas vesicles (Pfeifer 2012). G) Alpha carboxysomes; arrows indicate vertexes, triangle indicates RuBisCO (Schmid et al 2006).