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Review

Engineering design principles for organelle size control systems

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ABSTRACT

Organelle size is an important determinant of organelle function, and for this reason cells have evolved mechanisms to control and adjust organelle size in the face of intrinsic biological fluctuations. Size control systems have been found that employ a variety of distinct mechanisms, which fall into a small number of classes. Each class represents a design principle by which artificial size controllers could be developed for synthetic biology applications.

Contents

1. Introduction—controlling size as a design problem

Organelle size control is critical for proper cell function and architecture, but the control systems that a cell uses to regulate the size of its organelles remain completely unknown. Size controllers are inferred to be present whenever there is a structure, such as a flagellum or a membrane-bound organelle, that could in principle be of any size, but instead adopts a narrow distribution of sizes that is reproducible in a given cell type. The ability of a cell to restrict the size of an organelle to a narrow distribution with a cell type-specific mean value indicates size control. We will not, in this review, concern ourselves with size control for structures like ribosomes that self-assemble from an inherently fixed number of different subunits and thus never vary in size, nor will we consider the mechanisms that determine the size and growth/division rates of whole cells. Examples of biological structures for which size control has been studied include bacteriophage tail length [1], eukaryotic flagellar length [2], prokaryotic flagellar length [3], mitotic spindle length [4,5], actin thin filament length in sarcomeres [6] and stereocilia [7], and nuclear volume [8].

The precision with which organelle size is maintained varies from system to system. For eukaryotic cilia and flagella, the coefficient of variation (standard deviation divided by mean) of length generally falls in the range of 10–20%, with much greater similarity in length between multiple cilia within one cell than between cilia of different cells in a genetically identical population [9]. How does the cell impose this level of control on organelle size?

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2. Significance of engineering organelle size control

Organelle size control is not only a fascinating problem of basic cell biology, but it also has potentially significant applications. There is much current interest in learning how to build machines that can grow and assemble themselves using living cells or biological molecules as building blocks. Intracellular machines have two potential advantages. One is biological integration—the ability of cells to live and move within an intact organism such as a human patient, and to deliver a machine to the correct location within the body. A second advantage is self-production—by allowing cells to grow and divide, an intracellular machine can replicate exponentially, resulting in massively parallel systems orders of magnitude more complex than those that can be attained by current silicon-based technology.

Current synthetic biology progress has focused almost exclusively on constructing networks of diffusible regulatory molecules, with no defined spatial structure. Such small-scale networks can perform a remarkable array of computational, control, and timing functions [10–12]. However, to build "real" machines within cells, capable of mechanical operations or of linking identical computing units into large ordered arrays, we will need to control the spatial arrangement of structures. In principle one could imagine using optical methods to directly pattern structures within cells by some sort of direct-write technology. However, the true power of biological systems lies in their capacity for self-organization and reproduction. Having to directly write structures into individual cells, one at a time, would largely undercut the inherent power that could be gained from using cells to build machines. The key to unlocking the true potential of synthetic biology for building massively parallel devices is to learn ways to direct cells to self-organize machines of arbitrary complexity, without having to explicitly download structural information on a cell-by-cell basis. The natural mechanisms used by cells for building structures of defined size, shape, and position, could be harnessed for this type of purpose. We thus anticipate that by understanding how natural systems work, we can learn how to reprogram these systems to produce new types of structures. Moreover, we hope that by identifying the fundamental control modules that regulate the size, shape, and position of intracellular structures, we will be able to implement synthetic versions to produce intracellular structures of our own specification.

3. Multiple approaches to size control

A well-known thought exercise for undergraduate engineering students asks them to think up as many ways as possible to measure the height of the Empire State Building using a barometer. The obvious way is to measure the difference in air pressure at the top and bottom and calculate the height taking into account the density of air. Other ways range from the slightly less obvious (drop the barometer off the top of the building and time the fall) to the highly creative (offer to trade the barometer with the building manager in return for information about the height). The point of the exercise is that there are potentially a great many ways to measure the size of an object, even given a fixed set of tools, but the only design criterion that really matters is that the method has to work. The same situation holds in the evolution of cells. There may be many ways to use the molecular toolbox of a cell to measure organelle size, and the only constraint is that the method has to work well enough to provide a selective advantage during evolution.

4. Dynamic versus static structures

Before discussing different size control mechanisms, we must draw a distinction between two types of structures: static and dynamic [13]. Static structures are those which, once assembled, do not undergo any changes in composition or organization. Examples of static structures include brick houses and viral capsids. Dynamic structures are those which, even after assembly is apparently completed, continue to undergo turnover with loss of components balanced by addition of fresh components. One example of a manmade dynamic structure is a fountain, where maintenance of height requires pumping of new water to balance loss of water from the top of the fountain. Other examples in the manmade realm might include old fixer-upper houses that require constant construction just to balance ongoing decay. Most manmade structures tend to be static, for obvious reasons. In contrast, most biological structures are highly dynamic. Virtually all intracellular organelles undergo turnover. Even such apparently solid structures as bone are known to undergo continuous remodeling, even if the time scale is relatively slow.

The importance of distinguishing static from dynamic structures is that this distinction determines when the size control system must operate [13]. For a static structure, size control must be exerted during the time of initial assembly, and acts by turning off the assembly process when the structure has reached the appropriate size. For a dynamic structure, size control must operate continually, even after the organelle has reached is desired size.

5. Size control by quantal synthesis of limiting precursors

One simple way to control the size of a structure is to produce a precisely defined quantity of one or more size-limiting building blocks of the structure. For instance, if a contractor is provided with a fixed quantity of bricks, the size of the brick house he will be able to build is tightly constrained. Naturally, this mechanism does not solve the problem of size control, but simply changes it to a new problem—quantity control. However, controlling the quantity of a biological molecule falls within the realm of well-known mechanisms for measuring molecular concentration. Thus if quantity of a limiting precursor can be shown to be the key size control mechanism, it would immediately provide a starting point to looking for more detailed systems by exploring the transcriptional and translational control of production of individual precursor molecules.

Quantal synthesis of precursor as a size control component has been proposed for length regulation of cilia and flagella. The length of cilia in sea urchin cells appears to correlate with the quantity of a particular protein, tektin, that forms a building block of the outer doublet microtubules of the cilium [14,15]. Cells that produce more tektin have longer cilia, and cells that produce less tektin have shorter cilia. Unfortunately this result is simply a correlation—the key experiment would be to produce tektin under the control of an inducible promoter and show that changes in the expression level of tektin are sufficient to produce changes in length. In the flagella of *Chlamydomonas*, experimental evidence that one or more cytoplasmic precursor protein pools are limiting for length comes from measurements of flagellar regeneration following severing in the presence of protein synthesis inhibitors [16]. In such experiments, flagella grow back only to about half normal length. This shows that one or more proteins in the cytoplasm are used to build a new flagellum during regeneration. Unfortunately we do not know which protein out of the several hundred proteins that compose the cilium/flagellum is actually the one that limits the length of the structure. If candidate limiting precursor proteins were to be identified, however, they could be confirmed by placing their expression under control of inducible promoters.

The fact that distinct individual organelles are competing for a limiting supply of shared precursor molecules means that they are in competition amongst themselves. It is interesting to consider the extent to which ecological competition theory may be informative regarding organelle size control. At the very least it tells us that it may not always be sufficient to consider individual organelles in isolation, and that fully understanding the dynamics of size control may require an integrated model incorporating behaviors of multiple organelles. In the case of eukaryotic flagella, such competition has been dramatically demonstrated in the unicellular green alga *Chlamydomonas* by experimentally severing one of the two flagella. When this is done, the severed flagellum regenerates, but while it regenerates the other flagellum resorbs, presumably because its supply of precursor proteins is being choked off [16]. New improvements in laser ablation techniques should allow this type of analysis to be applied to other organelles, in order to test for this type of competition.

6. Size control by molecular rulers

A molecular ruler is a molecule, usually a protein, whose length matches the desired length of a structure whose size is under regulation. One typically envisions an elongated protein that adheres to the side of a growing structure, with one end of the protein attached to the initiation site of assembly of the structure, and the other setting the length at which assembly will terminate. The classic example of a molecular ruler is a protein whose length appears to set the length of the bacteriophage tail [1]. In this case, one end of the gene H protein associates with the initiation site of tail assembly, and the other end is thought to track the growing end of the tail, protecting it from a capping factor that would terminate assembly if incorporated. Molecular rulers have mostly been proposed for relatively small, prokaryotic structures including the injectisome [17] and the flagellar hook [18]. An example of a non-protein-based ruler is seen in assembly of tobacco mosaic virus, where the length of the RNA genome apparently sets the length of the assembled virus [19]. In eukaryotic cells, it has been proposed that the giant protein nebulin might act as a ruler to control actin filament length in sarcomeres, however this is still highly controversial [6].

Ruler-based size control systems suffer from two apparent limitations. The first is that the maximum length that can be set is limited by the maximum length of a protein. This limitation is less severe than one might think, since some proteins such as titin can be extremely long. Moreover, vernier-type mechanisms have been proposed whereby a pair of ruler proteins of unequal length would co-assemble side-by-side, such that their two ends would only line up when the combined assembly reaches a length given by the least common multiple of the two ruler lengths. So far there have not been any experimental reports of such a vernier mechanism operating in any known system. A second limitation is that the cell apparently loses the ability to change length of a ruler-regulated structure, since the length is inherent to the size of the ruler molecule. However, alternative splicing can produce rulers of differing length, and indeed this has been proposed to account for tissue-specific variation in actin filament length in sarcomeres, where length appears to correlate with the number of exons in different splice isoforms of nebulin [20].

7. Size control by time of flight measurement

Einstein was famous for presenting thought experiments involving the interplay between clocks and rulers. As a simple example, to measure the distance to some far-away landmark, just put a clock on a train, record the time displayed on the clock when the train leaves, and check its value when the train arrives. Assuming the train travelled at a constant known speed one can then infer the distance by reading the value on the clock. An even simpler scheme would be to use a kitchen timer, start the timer when the train leaves, and then check whether the timer has rung before the train reaches its finish. If so, it means the finish point is greater than a defined distance from the start.

The molecular equivalent of the kitchen-timer scheme would be to use an enzyme, such as a G protein, with a well-defined turnover rate, load it with a substrate, and then send it from one end of a cellular structure to the other. The status of the reaction could then be queried at the other end. For the case of a G protein, it would be loaded with GTP and then sent to the end of the structure for instance by attaching it to a motor protein. When it arrives, if it contains GTP bound it means the structure is not yet long enough, whereas if it contains GDP it means the structure is too long. By adjusting assembly or disassembly of the structure as a function of the GTP/GDP ratio of the arriving clock proteins, an arbitrarily precise control of size could be achieved. This type of mechanism is termed “time of flight” because the time required for a protein to traverse the organelle is used as a proxy for its length. A time of flight mechanism might be reasonable in the case of cilia and flagella, where there is a well-established kinesin-driven transport process termed intraflagellar transport (IFT) that moves a protein complex from the base of the cilium out to the tip and back again [21]. A clock protein attached to this IFT complex would be able to probe distance via a time of flight scheme. Interestingly, one of the proteins that composes the IFT complex is a G protein [22], so this might be a candidate clock. Whether or not this protein actually acts as a time of flight length sensor remains to be experimentally tested.

8. Feedback control by functional output

For organelles whose function depends on size, the cell could in principle regulate size by measuring the functional output of the organelle and adjusting size accordingly. The most promising example of such a mechanism is the unfolded protein response (UPR) which responds to levels of unfolded protein within the endoplasmic reticulum (ER). The UPR triggers transcription of many genes including some that encode enzymes needed for ER membrane synthesis [23], and when the UPR is activated it causes increased growth of ER [24]. The logic of the pathway is such that if the ER is too small, it will not be able to mediate folding of all the secreted proteins, thus triggering the UPR pathway. This, in turn, will stimulate production of new ER membrane leading to increase in ER size. A similar control of organelle size by function is seen in peroxisomes, where impairment of medium chain fatty acid oxidation, a key enzymatic activity of peroxisomes, results in enlargement of the peroxisomes [25], suggesting that the cell can recognize the functional deficit and attempts to alleviate the problem by increasing peroxisome size. In these cases, however, size increase is seen in pathological situations of induced functional deficit, and it is still not proven that the feedback from function to organelle enlargement plays a role in size control under ordinary circumstances.

The reason that functional output could, at least in theory, be used to control size is that the function of an organelle depends on its size, so that any measure of organelle function could act as an indirect indicator of size. This would be true of any output derivable from the organelle, even one not related to its primary function, as long as that output was dependent on organelle size. For example, it has been proposed that calcium channels in the membrane of the flagellum could provide a calcium current that would be proportional to the length of the flagellum [26]. Such a model would require a pathway that responds to the calcium cur-

rent and that, in turn, modulates either the assembly or disassembly of the flagellum.

9. Size control by dynamic balance mechanisms

Most of the mechanisms discussed thus far involve one or more additional components superimposed onto the basic machinery of organelle biogenesis in order to regulate size. However there is a class of models in which no additional control components need be invoked. Most organelles are dynamic, undergoing continuous turnover in which constant assembly is required to balance constant disassembly. If either the assembly or disassembly process is inherently size-dependent, this could potentially suffice to dictate steady-state organelle size. Consider a membrane-bound organelle, for example the Golgi apparatus, that exchanges vesicles with a large pool of membrane, for example the ER. For this hypothetical case we ignore other vesicle trafficking events and just consider exchange of the organelle with the ER. Vesicles continuously bud from the ER surface, move out to the organelle, and then fuse with it. At the same time, vesicles are constantly budding from the surface of the organelle, moving back to the ER, and fusing with it. Every time a vesicle fuses with the organelle, the organelle surface area increases by some small amount, and every time a vesicle buds off of the organelle, the organelle surface area decreases. Suppose the anterograde vesicles (i.e. the ones moving from ER to the organelle) have surface area S_a, and the retrograde vesicles returning to the ER from the organelle have surface area S_r. Suppose furthermore that vesicles bud from the ER at some constant rate k_a per unit time. It is reasonable to assume that k_a is independent of the size of the organelle. In contrast, the rate of budding of vesicles from the organelle is likely to be dependent on the organelle surface area, since more surface area means more sites at which a bud can initiate. The simplest dependence would be that the total rate of vesicle budding off of the organelle is given by $k_r S_o$, where S_o is the surface area of the organelle. Under these assumptions, there is only one stable steady-state value possible for the organelle surface area, given by $S_o = k_a S_a / k_r S_r$. This mechanism would produce stable size control without requiring any additional feedback machinery or rulers, relying instead on the intrinsic surface area dependence of the vesicle budding process. This is a purely hypothetical example but is presented to illustrate the concept. Size control mechanisms based on a dynamic organelle whose size is the balance of assembly and disassembly, where one process or the other is inherently size-dependent, will be termed a dynamic balance mechanism.

Dynamic balance appears to be a likely mechanism for controlling size of cilia and flagella. Analysis of flagellar microtubule dynamics showed that the outer doublet microtubules that form the structural scaffold of the flagellum undergo continuous turnover at the distal end of the flagellum [27]. It was further shown that the continuous removal of tubulin from the tip occurs at a rate that is length-independent, while the addition of tubulin to the tip relies on intraflagellar transport [27]. IFT is a kinesin-based motile process by which protein complexes called IFT particles are moved to the tip, carrying flagellar building blocks such as tubulin [21,28]. The fact that the quantity of IFT protein within the flagellum was found to be length-independent [27,29] suggested a very simple model for length regulation in which as the flagellum grows longer, the IFT particles have to travel over increasing distances, thus taking longer to deliver their cargo. Since the total number of IFT particles stays constant, this means that IFT becomes less efficient as the flagellum grows, such that at a single critical value of length, the rate of IFT-dependent assembly at the tip will precisely balance the rate of length-independent removal from the tip. This length represents the steady-state length of the flagellum. This length control model does not require any explicit length sensor and can account for all published data on the flagellar length control process [2,27,29].

10. Probing size control mechanisms

How can one decide which of the various types of size control mechanisms might apply to a given organelle? In general, genetic approaches provide an unbiased approach to exploring size control systems. Genetic screens have revealed genes involved in size control of many organelles including cilia/flagella [30–32], vacuoles [33,34], lipid droplets [35], and peroxisomes [36]. However, merely identifying a set of genes does not in itself tell us anything whatsoever about how the size control system works. One should thus be extremely careful not to confuse a nice-looking genetic pathway diagram with a mechanistic understanding, despite the visual similarity with an electrical circuit schematic diagram. Mechanistic understanding requires that we go beyond enumeration of gene-lists, by proposing and testing hypotheses about how the size control system might actually function. In this section we will review several generally applicable methods for probing organelle size control systems.

The total abundance of an organelle depends on two facets: size and number. For a given quantity of precursor, a cell could have either a large number of small organelles or a small number of large organelles. This provides a way to probe size control, by manipulating organelle copy number and measuring how average organelle size varies with number. Consider for example the mechanism of size control by quantal synthesis of a limiting precursor. This model predicts that organelles sizes, listed as a function of copy number, should form a geometric series. That is, when the number of copies of an organelle is k, the average size should be proportional to $1/k$. For example, when twice as many organelles are present, they should all be half as big, and if four times as many are present, they should be one fourth as big. This is a simple prediction to test, and only requires a way to vary organelle number. In most cases, our understanding of how organelle number is controlled is even less well-developed than our understanding of how organelle size is controlled. Fortunately we can sometimes use genetic mutations that alter organelle segregation during mitosis to produce variation in number [37]. This approach has been applied to exploring flagellar length control in *Chlamydomonas*, using mutants defective in mitotic centriole segregation, with the result that flagellar length depends much less strongly on number than predicted by the quantal synthesis of limiting precursor model [29,38].

Another general type of measurement is growth rate versus size. Different size control systems predict different dependencies of growth rate on instantaneous size. For example, a standard molecular ruler model would predict that growth would occur at a constant rate until a critical size was reached, at which point growth would abruptly stop. In contrast, balance point models generally predict that growth is a continuously decreasing function of size, and the nature of the functional dependence of rate on size can give important clues as to mechanism. For instance, if it was found that the net growth rate of surface area of a membrane-bound organelle was a linearly decreasing function of surface area, this would directly support the vesicle exchange mechanism discussed above. The advantage of this type of measurement is that it is simple to execute, one simply needs a way to observe growth or shrinkage of an organelle as a function of its current size. This is most conveniently done when an organelle first forms. Measurement of flagellar growth rate during flagellar regeneration in *Chlamydomonas* has confirmed the predictions of the balance point model [29].

Feedback control systems are often invoked for size control, but they can be rather difficult to test. This difficulty arises from the fact that one can, in general, mimic the function of any open-loop

control system with a feedback control system, hence it can be difficult or impossible to devise a phenomenological test that would allow one to rule out a feedback system. Rather, testing feedback requires us to first identify possible components of the feedback loop. An important point, however, is that merely identifying a signaling molecule involved in size control does not by any means prove that it is operating within a feedback loop. Cells change the length of the cilia and flagella under a variety of circumstances, and such changes are surely mediated by signaling pathways. Mutations in such pathways might result in changes in steady-state length, hence one cannot know, just by identifying a length-regulating kinase, whether this kinase acts within a feedback system or simply provides external input to the system. The only way to determine whether or not a signaling molecule acts within a feedback system that regulates organelle size is to measure the activity of the molecule in vivo and ask whether it changes in response to alterations in size. Moreover, since only negative feedback loops can provide stable size control, one can impose a further condition on the test, in that the activity of the protein must vary as a function of size in the proper sense, i.e. increasing or decreasing as size increases or decreases according to the requirements of negative feedback. Consider the example of LF4 kinase. Null mutations in LF4 result in elongated flagella [30], hence the normal function of LF4 must be to promote reduction in size. If LF4 is acting within a negative feedback loop, then its kinase activity should be increased when flagella are elongated, and decreased when flagella are shortened. The test, therefore, is to modulate flagellar length and measure the effect on LF4 activity. One caveat with this type of test is that the perturbation used to modify length within the test must not act directly on a component of the feedback control pathway. This may be hard to enforce if the bulk of the proposed pathway is unknown. This represents one situation in which determination of a complete genetic pathway using saturated screens is of direct importance in testing mechanisms.

11. Prospects

Thus far, studies of size control have been most effective when focused on simple organelles with linear geometries. Now that multiple possible mechanisms for controlling organelle length are known, it is important to begin more detailed analysis of size control in more complicated geometries. This will certainly require development of automated quantitative image analysis tools to allow size to be rapidly determined, preferably in vivo. For any given organelle, one must not only be able to measure size, but also to perturb it, and this will require development of new genetic and chemical tools to manipulate organelle size. Finally, in order to achieve true mechanistic understanding, it will be critical to move beyond hand-waving types of arguments and develop predictive, quantitative computational models that can be used to test whether a given size control system model can adequately account for measurable behaviors of size control systems.

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