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Protocell design through modular compartmentalization

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De novo synthetic biological design has the potential to significantly impact upon applications such as energy generation and nanofabrication. Current designs for constructing organisms from component parts are typically limited in scope, as they utilize a cut-and-paste ideology to create simple stepwise engineered protein-signalling pathways. We propose the addition of a new design element that segregates components into lipid-bound 'proto-organelles', which are interfaced with response elements and housed within a synthetic protocell. This design is inspired by living cells, which utilize multiple types of signalling molecules to facilitate communication between isolated compartments. This paper presents our design and validation of the components required for a simple multi-compartment protocell machine, for coupling a light transducer to a gene expression system. This represents a general design concept for the compartmentalization of different types of artificial cellular machinery and the utilization of non-protein signal molecules for signal transduction.

Impressive feats of synthetic biological engineering have been demonstrated by modifying the metabolism of living organisms [1–6], altering enzyme localization in metabolic pathways to improve efficiency [7,8] or sensory component addition [9]. Such designs, however, build upon existing organisms, rather than engineering from component parts. As a consequence, they often suffer from inherent complexities, such as the required regulation of the new metabolic processes [10]. Novel, simpler machines therefore offer an attractive alternative: the bottom-up approach enabling construction of self-regulating synthetic machines [11–13] utilizing repositories of parts such as 'biobricks' [14] in the absence of an existing biological system. This imparts advantages including elimination of unpredictable interference by the host organism metabolism [15], removal of host transcriptional gene regulation complexity [16] and the potential addition of regulatory elements or external controls that switch on the activity of the protocell machine [17]. Additionally, there is potential for addition of componentry using soluble nanolipoproteins [18] that allow the incorporation of incompatible systems without the requirement for spatial segregation. Despite the advantages of de novo synthetic biological design, the development of such systems has been limited: benefits of reduced complexity come with disadvantages including loss of control fidelity and component incompatibility. We present an experimental design that could illustrate a more general strategy for incorporating control elements without significantly increasing design complexity, which involves linking modular compartmentalized elements (proto-organelles) via non-protein-signalling molecules. This mimics the utilization of multiple signalling modes in living cells and allows for the incorporation of numerous potential subsystems, potentially including apparatus for protocell budding [19], leading the way towards protocell replication [20], liposomal biomachinery encapsulation [21], protein–protein communication that is either membrane mediated [22]

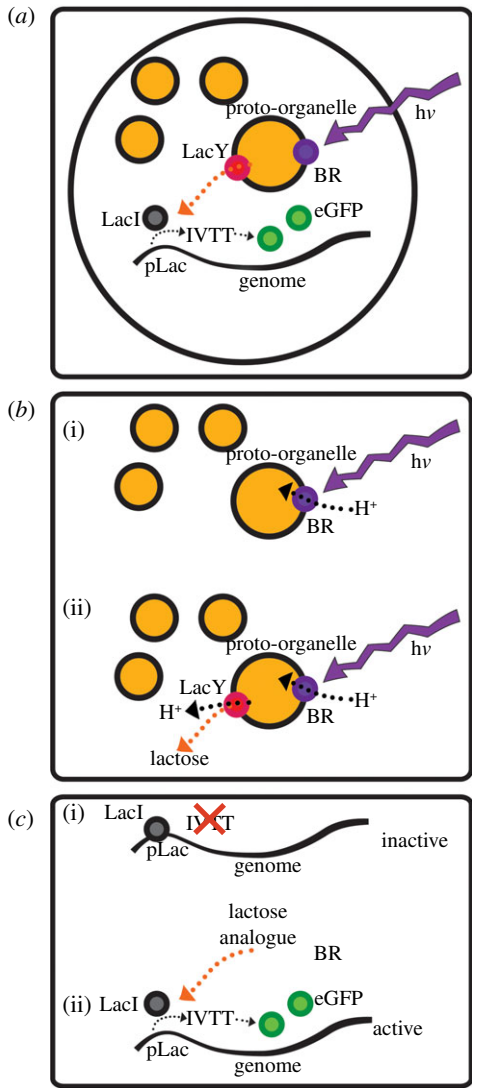

Figure 1. (*a*) Design and components of the light-sensing protocell featuring the light-sensing proto-organelle subsystem and the fluorescent molecular readout module. The entire complex is encapsulated and stabilized by a lipid monolayer of diphytanoyl phosphatidylcholine in hexadecane for measurement. (*b*) The light-sensing proto-organelle subsystem design. (i) hν powers acidification of proto-organelles by BR. (ii) The resulting H^+ gradient provides energy for H^+/lactose symport via LacY coupling lactose release to light sensing. (*c*) The fluorescence molecular readout module. Upper: *the inactive state*—transcription and production of eGFP is prevented by association of the repressor LacI to the pLac control region of the artificial genome. Lower: *the active state*—the lactose analogue (IPTG) results in release of LacI from the pLac region and removal of transcriptional repression resulting in eGFP production.

or mediated through direct interaction, energy production modules [23] and genome replication modelled after retroviral systems [24].

Figure 1*a* presents our modular compartmentalized lipid-stabilized protocell design including: (i) a light-sensing lipid-stabilized proto-organelle, which in response to light generates a H^+ gradient and triggers lactose release, and (ii) a fluorescent molecular readout module, which is a molecular signal transducer that links the output signal (the presence of lactose analogues) to protein expression. Our design segregates protocell components into subsystems, thus allowing additional levels of complexity and control to be realized, and highlights future opportunities for facilitating the development of semi-synthetic molecular machines.

The first subsystem is a light-sensing proto-organelle comprising a bacteriorhodopsin (BR) light sensor and a lactose permease (LacY) signal transducer incorporated into liposomes, constructed from purified 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) lipids, that exports a control molecule (lactose) in the presence of a light signal. Illumination of BR acidifies the interior of the proto-organelle by light-driven pumping of H^+ across the lipid membrane. The H^+ gradient leads to LacY-driven co-transport of H^+ and lactose from the proto-organelle interior to the exterior (figure 1*b*). This proto-organelle is housed within the second subsystem: the fluorescent molecular readout module. This module consists of a simple 'genome' containing enhanced green fluorescent protein (eGFP) with engineered control sequences that are repressed by the presence of the transcriptional repressor, LacI. Increasing the concentration of a lactose analogue signal molecule transduces the incoming light signal to this subsystem by repression removal. In combination with all the components required for *in vitro* transcription and translation (IVTT), this forms a complete system for synthesis of mRNA and consequent eGFP production (figure 1*c*).

Figure 2 shows the measured activity of our protocell modules. Light energy drives H^+ pumping into the liposome interior using the light-powered proton pump, BR. We monitored the resulting acidification of the liposome interior via changes in fluorescence excitation of the pH-sensitive dye pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid) [25,26]. The observed changes in the ratio of the 450/405 nm fluorescence excitation bands are indicative of liposomal pH changes (see the electronic supplementary material, figure S1) and therefore showed that we could create a H^+ electrochemical gradient in our proto-organelles. This was demonstrated by the acidification of liposomes containing functional BR following illumination (figure 2*a*). We added capability for signal molecule (lactose) release from the interior of the organelle to the external solution: coupling BR activity to signal release through LacY. Utilizing lactose as the signal molecule allowed very sensitive detection via a linked chemiluminescent assay. Figure 2*b* shows the result of illumination of the BR and LacY proto-organelles and the light-driven lactose release over time. Illumination doubles the concentration of lactose released from proto-organelles compared with non-illuminated under identical conditions. These observations validate our initial subsystem design (the proto-organelle light sensor), which consists of only five different molecules (BR, LacY, DOPE, DOPC and lactose), excluding the buffer components.

The readout module required the development of a control mechanism capable of responding to the incoming signal molecule (lactose or lactose analogue) from the light-sensing proto-organelle. We designed a controllable switch that is able to turn on mRNA production to express eGFP in the presence of a signal molecule (figure 1*c*) monitored by fluorescent readout. We used the lac operon to repress expression, specifically mRNA production in the presence of the LacI repressor protein that tightly associates with a pLac control region, which we designed into our linear eGFP 'genome'. This repression only occurs in the absence of a chemical control signal (in our case, the lactose analogue isopropyl β-D-1-thiogalactopyranoside (IPTG): a non-metabolizable lactose analogue that unlike lactose does not require an additional enzymatic isomerization step for LacI interaction: this efficiently binds to LacI and removes repression).

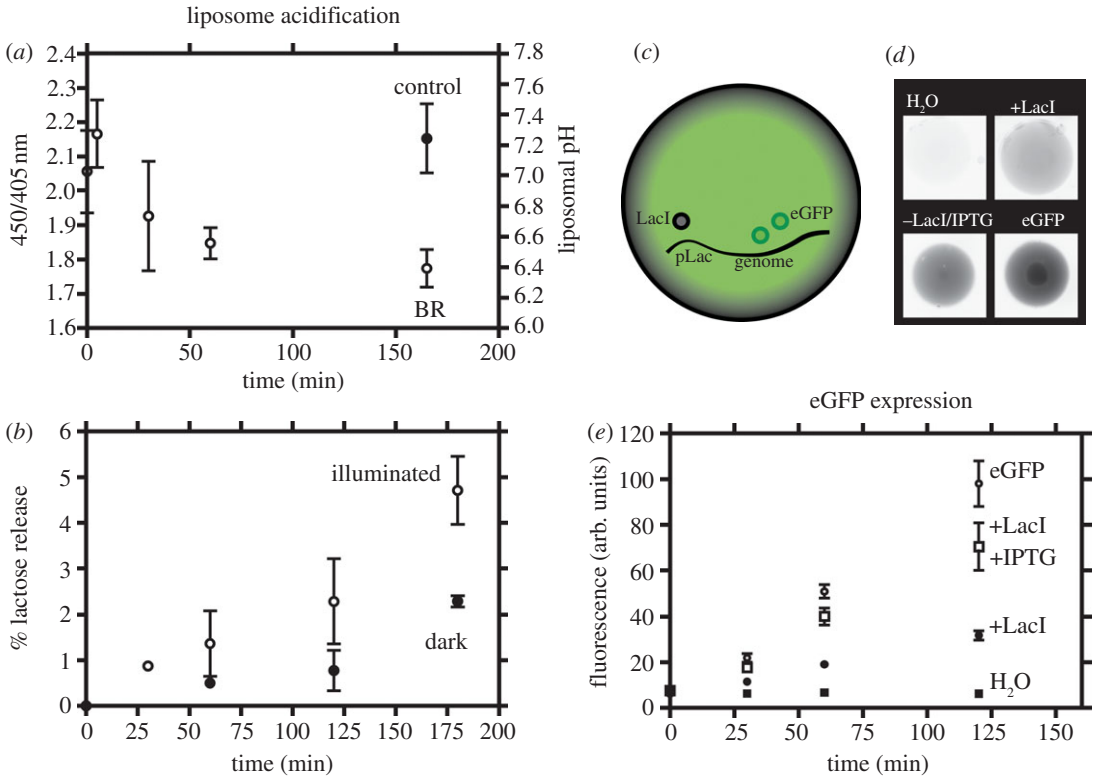

Figure 2. Activity of the individual protocell components. (*a*) Acidification of the proto-organelle subsystem (BR liposomes) following illumination, as determined by pyranine fluorescence. Calibration for the liposomal pH is shown in the electronic supplementary material, figure S1. (*b*) Release of lactose from LacY and BR containing proto-organelles illuminated (open circles) and non-illuminated (filled circles). (*c*) The fluorescence molecular readout module. (*d*) Representative fluorescence images of eGFP expression in lipid-stabilized protocell drops (inverted colour for clarity). (*e*) Quantification of eGFP expression over time. All samples contain the eGFP 'genome' with the exception of the H_2O control; +LacI contains LacI; +LacI/IPTG contains both LacI and IPTG; and eGFP contains neither LacI nor IPTG. Errors are calculated as s.d. from three repeat experiments. In cases where error bars are not shown, this is because they are smaller than the symbols.

The design of our fluorescent molecular readout module subsystem is shown in figure 2*c*, and the resulting protocell solutions and eGFP expression in the presence and absence of the lactose analogue signal molecule and the LacI repressor in figure 2*d*. We measured the fluorescence of our protocells as sub-microlitre lipid-stabilized protocell drops in hexadecane allowing for the isolation of individual protocell drops. Fluorescence was negligible in the absence of an eGFP genome (see examples of raw data in figure 2*d* and quantified fluorescence in figure 2*e*). In the presence of the eGFP genome, eGFP fluorescence increased over time, consistent with efficient induction of expression. We verified that LacI could turn off this expression with eGFP production, determined by the fluorescence, decreasing to approximately 30% of that observed without repression (figure 2*e*). We then determined whether we could switch expression back on with the addition of the lactose analogue signal molecule (IPTG). Upon inclusion of the signal molecule in the protocells, repression by LacI was relieved, resulting in efficient eGFP production up to approximately 75% of that observed in the absence of any repression. This therefore demonstrated our ability to construct a system that can be readily switched, from an 'off' to an 'on' state, in the presence of the lactose analogue and the repressor LacI. This validates our fluorescent molecular readout module subsystem design and demonstrates the feasibility of the components used here for signal transduction between modules via a small molecule and associated protein componentry.

Designing protocell organisms from relatively simple components has potential advantages over the modification of biological systems, in particular the ability to predict the behaviour of the system under design *a priori*. The development of these systems will require both the segregation of components and the addition of external regulatory control elements, for example by isolating control elements in lipid-stabilized proto-organelles that can then be integrated into a signal-response element via non-protein signal molecules. This ideology allows the creation of simple synthetic systems, albeit with complex behaviour, utilizing a minimal component count. The resulting designs offer system complexity while avoiding the need to engineer existing life forms, which has inherent disadvantages such as unwanted side reactions and unanticipated changes to host metabolism [27], inhibitory regulatory control mechanisms that limit function and inherent genetic noise present in natural systems [28,29]. We have validated each of these subsystems, such that the modules can be combined as outlined in this design brief or inserted into other protocell frameworks leading to new synthetic biology machines that are controllable and will respond to environmental conditions. Future design iterations will build upon our development platform, incorporating otherwise incompatible elements together and using a high fidelity of regulation through the addition of further control elements. Such designs, where individual components can be segregated but linked by signal molecules, will provide additional control complexity to synthetic biological designs with potential applications in the construction of synthetic bioreactor machines that respond to external switches to control internal processing. Although not an issue with the protocell volumes we have utilized, in small volume systems, some consideration has to be made

for the stochastic distribution of componentry, which can influence system behaviour [30]. The compartmentalized and modular design template reported here will act as the foundation for the further sequential addition of independent modules, with multiple independent input and output signals, resulting in a rapid increase in the complexity of future protocells that can be engineered.

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