A ‘bioproduction breadboard’: programming, assembling, and actuating cellular networks

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Abstract

With advances in synthetic biology and biofabrication, cellular networks can be functionalized and connected with unprecedented sophistication. We describe a platform for the creation of a ‘bioproduction breadboard’. This would consist of physically isolated product-producing cell populations, product capture devices, and other unit operations that function as programmed in place, using unique, orthogonal inputs. For product synthesis, customized cell populations would be connected through standardized, generic inputs allowing ‘plug and play’ functionality and primary, user-mediated regulation. In addition, through autonomous pathway redirection and balancing, the cells themselves would provide secondary, self-directed regulation to optimize bioproduction. By leveraging specialization and division of labor, we envision diverse cell populations linked to create new pathway designs.

Keywords

Microfluidics; dynamic gene expression; consortia; signaling; pathway engineering

Introduction

There is an emerging technological focus towards biologics production via small scale systems comprised of linked component parts. By balancing customizability and standardization through connected ‘pods’, these modular systems feature shorter fabrication and assembly time, reduced cost, less waste, and increased flexibility [1]. On a laboratory or process development level, this practice in compartmentalized operations is captured by the use of microfabricated devices as process scouting devices (PSDs), essentially providing a first draft of the process development pipeline [2]. Separately operational but physically linked PSDs can serve to improve upstream production processes and downstream purification processes [3]. We anticipate, however, that by incorporating advances in genome editing, genetic circuit design, dynamic gene expression and even task-distributing microbial consortia, these concepts could be applied to pathway engineering for the creation of as yet undiscovered pathways for product synthesis.

Analogous to electronic circuit elements that are spatially insulated and electronically connected, microfabricated devices can physically isolate biological elements and connect them through fluidics. As the device interface between biology and electronics becomes more pliant [4, 5], the conferred advantages of microfabricated devices has led to the expansion of lab-on-a-chip, organ-on-a-chip and even human-on-a-chip devices (reviewed by [6]). In many of these systems, however, biological pathways and biomimetics are used to test pharmacokinetic properties of candidate drugs when exposed to various cells or tissues. We note the advantages of spatial isolation, precise control over fluidics, and low volume requirements can also be exploited in the field of pathway engineering for biologics production.

A Bioproduction Breadboard

We envision a ‘bioproduction breadboard’ (see graphical abstract) with spatially controlled biological components (e.g., cell populations, effector proteins, genetic vectors, etc.) that dynamically interact as interchangeable parts for the generation of a variety of different compounds. Each biological component functions independently, but are assembled together to perform a pathway-on-a-chip. As illustrated in the example in **Figure 1**, these cell populations would be interchangeable, where a feedstock of lignocellulose could be fed to two different cell populations to produce two different products.

The ‘bioproduction breadboard’ would use generic and specialized signaling systems to alter an individual module, or to change the connection between modules. By the inclusion of orthogonal inputs that eliminate unwanted crosstalk, the on-chip cell populations can be ‘customized’, or inserted at spatially isolated locations with programmed function. Diverse cell populations would potentially be connected via a ‘standardized’ biological basic input/output system (bBIOS) using a few generic signals (e.g., electrons, photons, metabolites) as inputs that are recognized by genetic controllers enabling simple ‘plug and play’ connectivity.

Control of the bioproduction operations would be bilayered: user-mediated genetic regulators and cell-mediated dynamic expression. Genetic regulators provide the primary means for control, and there has been considerable progress in the construction of more sophisticated, multi-functional, robust, and reliable circuits. Artificial gene circuits have increased progressively in terms of functionality, incorporating externally-actuated switches, oscillators, logic operators, filters, and biocomputers (reviewed by [7]). Also, building on robust input modalities, cell-based autonomous controllers are constructed for dynamic real-time control of

gene expression based on prevailing cues. These provide a secondary layer of regulation, where cells autonomously redirect and optimize their own biological pathways, as designed.

Recognizing the incorporation of many heterologous genetic components within a single cell can impair its metabolic activity, synthetic cell consortia are being examined wherein subpopulations are pieced together for assembly of complex function. This avoids diminished activity by distributing function among less-affected, yet nearby cells. These ‘pathway engineering’ contributions can be pieced together from component parts; assembled and connected in microfabricated devices to produce needed products.

In the following sections, we review three thrust areas that have contributed significantly to this goal and where additional advancements are needed: i) the development of signaling and ‘switching’ modalities within and between spatially segregated cell populations; ii) stimuli-responsive gene expression controllers; and iii) the assembly of components, including synergistic microbial consortias, within spatially localized but connected locales. The complete breadboard would include product separations, purification, and perhaps delivery modules, but the focus of the current review is on product synthesis.

Part 1: Cell signaling and switching mechanisms

We first review development of signaling modalities for controlling pathways within cells and among connected populations. To achieve ‘plug and play’ flexibility that functionally links customizable, isolated cell populations, the ability to coordinate and connect across multiple, diverse populations requires signaling standardization. We address three signaling modalities that are uniquely suited to a ‘bioproduction breadboard’: light (photons), electrons,

and molecular cues that are biologically orthogonal to the embedded cells. These form the basis for a biological BIOS.

The flexibility of optogenetics allows light-mediated activation [8]. The first use of light to control transcription within synthetic biology employed phytochromes, two component systems comprised of an extracellular sensor and an intracellular response regulator [9] within *E. coli*. This led to an edge detection system [10]. Also, Olson and coworkers used a two-component system (TCS) in *E. coli* that responded in a dose-dependent manner to effector light intensity [11]. Subsequent optimization dramatically reduced leakiness and the dynamic range was increased over 100 fold [12]. As over 75000 TCSs have been identified [13], this platform, properly engineered and applied, represents a promising modality for stimulating genetic control elements within a variety of cell types. Further, through optically transparent calcium alginate hydrogels, cells can be aligned and assembled onto microdevices enabling localized cell placement and optical interrogation [14]. Through its versatility in actuation and readout, optical control provides a powerful tool to connect cell populations.

Another method for coordinating distinct cell populations borrows from native signaling processes already prevalent, particularly among bacteria. The gratuitous inducer, IPTG, serves as one of most common examples of an orthogonal molecular signal in that it serves no function beyond actuating *lac* based controllers. While many signaling systems have been engineered to coordinate gene expression [15], the bacterial quorum sensing (QS) autoinducer, AI-2, that is produced by *E. coli* and scores of other species [16] might serve as an orthogonal signal, particularly after appropriately engineering its transduction mechanisms, as its genetic parts enable species-specific recognition [17]. The generation of the signal can be device-born [18, 19] and evidence suggests its concentration can be ‘programmed’ *in situ*. Also, by design and

construction of ‘controller’ cells, regulation of both signal AI-2 input and decay are possible, so that well-defined chemical inputs can be developed for controlling producer cells [20•]. That is, organized cell subpopulations could be tuned to respond in predetermined ways for the synthesis of novel products. Autoinducer-2 and other QS systems are thus natural choices for chemically-connecting cell populations as long as the signals themselves can be deployed with spatially defined and programmable means.

Also, electrical signals, in contrast to optical and chemical signals, would greatly increase speed and increase compatibility with electronic devices. Illustrated in **Figure 2**, Gordonov and coworkers electrically assembled and tuned biosynthetic enzymes on a chip, providing the first example of microelectronic and biological control of cell signaling using electrical signals [21••]. They showed a means to electrically modulate the generation of AI-2 and the subsequent QS-mediated control of cell phenotype. While not electrically modulating cells directly, altering the chemical signaling can lead to ‘quantized quorums’ based on input signal concentration [22].

As diverse cell populations are linked together in a ‘bioproduction breadboard’, the dynamic ranges of input functions and output products can be tested and optimized. Then, signaling modalities including photons, electrons, and appropriately aligned small molecules will provide means to monitor and control cellular systems for biologics production.

Part 2: Dynamic control of biosynthetic flux

Through user and cell-mediated control, the ‘bioproduction breadboard’ would consist of both primary and secondary regulation of the on-chip pathway. First, to establish and focus the cell’s biosynthetic activity on the desired objectives, biologically orthogonal signal inputs must minimize unwanted crosstalk among *intracellular* biological elements. That is, extracellular

signaling modalities must connect to intracellular controllers as ‘primary’ regulators and these connections need to be sufficiently isolated, avoiding unintended effects. Second, a ‘bioproduction breadboard’ is potentially user-initiated through light, electrons, or small molecule signaling that links to these primary regulators, but owing to the need for dynamically guiding what would be very complex cell behavior, a ‘secondary’ control mechanism that is autonomously operated may be advantageous. We review advances in systems that could be easily adapted to the ‘bioproduction breadboard’ to enable both levels of regulation.

Genetic switches – primary regulators

Genetic switches are noted for their relatively rapid response times, simple programmability, component modularity, and by the existence of computational tools for their design, assembly, and characterization. In particular, owing to their small size, relatively insignificant collateral effects on cells, and the diversity of their structures, we highlight advances in riboregulation [23] as primary regulators that could be connected to robust extracellular signals discussed above.

A significant step in orthogonal control was the development of *cis*-repressing and *trans*-activating riboregulators [24]. The orthogonality of this system was demonstrated as a ‘genetic switchboard’ that can independently and tightly regulate multiple genes in parallel [25]. A ‘genetic switchboard’ is defined as a system of genetic switches that link together biological pathways. By using four orthogonal synthetic riboregulators, Green and coworkers programmed user-mediated shunting of carbon flux through different pathways. More recently, riboregulator ‘toehold switches’ expanded the sequence space of riboregulators, thereby increasing the number of orthogonal inputs [26•]. Orthogonality had previously been limited in that trigger *trans*-acting RNAs had to contain an RBS sequence to release the repressing sequence. By sequestering most

of the *cis*-repressing riboregulator in a hairpin by the start codon and exposing only a small ‘toehold’ region, a *trans*-activating RNA can trigger translation with an arbitrary sequence. Further, *cis* and *trans* interactions were engineered with improved kinetics, increased dynamic range (400-fold), and the simultaneous regulation of up to 12 genes. Similar to riboregulators, small transcription activating RNAs (STARs) have been developed with multiple orthogonal signals and high dynamic range [27, 28]. These RNA based systems allow precise, user-mediated control of specific genes without any unwanted crosstalk; essentially cell population can be ‘customized’ by the user.

Autonomous systems – secondary regulation

Metabolic pathways connected through multiple cell populations would be connected and interconverted by the user, but these pathways could be self-regulated. *Autonomous* pathway redirection and pathway balancing are becoming pillars of dynamic gene expression (**Figure 3**) [29]; both approaches require tight and rapid responses to changing environments [30].

Autonomous pathway redirection is based on a cell sensing the accumulation of a particular intracellular signal signifying that it is the right time to switch on product synthesis. Autonomous pathway balancing uses the accumulation of intermediates to automatically adjust pathway redirection in a continuous manner. Recognizing the contribution of recent reviews [29, 31], we discuss exciting new work in both of these areas.

Pathway redirection

Pathway redirection is often used to switch from cell growth phases marked by biomass accumulation to product synthesis. The first example of the autonomous control of pathway redirection was in the production of lycopene, using acetyl phosphate (ACP) as a signaling

molecule [32]. ACP had been shown to indicate glucose availability, and when used as a switch, significantly increased lycopene production in *E. coli*.

While there has been significant work using pathway redirection, where the accumulation of an enzyme is used to redirect flux from biomass towards product synthesis [33, 34], the accumulation of a QS metabolite to redirect protein synthesis is a notable addition [35]. The secretion and uptake of QS molecules enable independent monitoring of the cellular activity as well as potential for actuation. For example, it was shown that the ‘metabolic burden’ of heterologous protein production is communicated through the QS signal AI-2 [36], and Tsao et al. leveraged QS to create an autonomous protein production system using AI-2 as a metabolic controller [35]. Liu et al. later used QS to generate useful biofuels such as 1,4 butanediol, where the production and cell growth phases are separated [37]. Soma et al. have also developed a QS-triggered metabolic toggle switch for transitioning from cell growth dominated pathways to the production of isopropanol [38].

Pathway balancing

From a control theory perspective, novel approaches are needed to develop reliable cell populations that continuously adapt pathway regulation to changing environmental conditions [39]. Recently, Dahl and coworkers developed dynamic pathway regulation of the mevalonate-based isoprenoid biosynthetic pathway, which led to improved yield of the isoprenoid amorphaadiene [40•]. Farnesyl pyrophosphate (FPP) is a toxic intermediate metabolite that accumulates in *E. coli* during isoprenoid biosynthesis. With no known FPP-responsive promoters, transcript profiling (RNA-seq) was used to determine endogenous *E. coli* promoters that respond to FPP. After characterizing activators and repressors, they use both up-regulating responsive (FPP consumption) and down-regulating responsive promoters (FPP biosynthesis), to

show increased autonomous production of amorphadiene. Analogously, Xu and coworkers developed a dynamic regulatory pathway for malonyl-CoA [41]. They engineered hybrid regulators that acted as both activators and repressors, and linked them to the pathways for cell growth and product formation resulting in improved fatty acid yield.

These examples of autonomous pathway redirection and balancing involved transcription, and translation; once translated proteins are assembled however, they in turn, last for hours. While on-off RNA controllers meet design objectives, they are not ideal for continuous dynamic control. Linking these systems to tunable protein degradation [42], where proteins deplete at a pre-determined rate would greatly improve control. Also, protein switches [43] that are tailored for real-time molecular actuation of enzymatic activity would be highly advantageous in that they could reside dormant in the cell until a metabolite cue reaches an actuating threshold (reviewed in [44]). Further, additional computational methods will be needed to incorporate the complexity of the dynamics of regulatory circuits, intracellular metabolites, and effector proteins, to engineer reliable cell populations [31].

Part 3: Cell consortia

Thus far we have highlighted design aspects of several promising genetic circuits deployed inside a single cell. While a stripped down product-producing ‘minimal cell’ concept has advantages [45, 46], enabling increased programming power and capability comes at a metabolic cost eventually reaching a point where a homogenous cell population no longer has the capacity to perform the required task. Further, with the division of labor and specialization, it may be more appealing to use a multicellular approach over a unicellular one (**Figure 4**). Engineering of specialized cells offers an atypical view of ‘plug and play’ in that it consists of a programmed consortia [47]. Benefits by using ‘connected’ cell populations include biological

‘noise’ reduction, as intercellular signals between cell populations can enable a more coordinated response and reduced stochasticity [48]. In addition, cells of vastly different capability can be linked, enabling far more complex biosynthesis reactions [49]. That is, vastly divergent and novel pathways could be arranged between organisms that are otherwise incompatible.

While the advantages of specialization are clear, microbial consortias have been limited in applications for bioprocessing because of the difficulty to concurrently control their composition and function [50]. The spatially controlled environment of a microfluidic device is ideal for these challenges as it is constructed in microscale, characterized by well-defined flows and concentration gradients, small sample volumes, and small cell numbers. In a sense, it relaxes the need to balance growth rates between two distinct populations [51]. With early successes in microbial consortia included the engineering of cell-cell communication [52, 53] and programmed cell death [54], the concept of spatially organized microbial communities for the production and break down of complex metabolites was advanced over ten years ago [55]. We will highlight recent advances that may be adapted into a ‘bioproduction breadboard’.

There have been many notable examples of synthetic microbial consortia constructed for the production of products, including sugar nucleotides, oligosaccharides, and biofuels (recently reviewed [56]). For example, Shin and coworkers genetically engineered two strains of *E. coli* to process xylan into ethanol [57]. Two hemicellulases were co-expressed in one strain to hydrolyze xylan into xylooligosaccharides. The second strain was engineered to import xylooligosaccharides and produce ethanol. Through the division of labor, the co-culture delivered a theoretical yield of ethanol of 55%. This case is a notable example for the relatively simple transition towards microfabricated devices, should the application be appropriate. That is,

due to growth rate differences, the two strains were cultured separately and combined after induction. Conceivably, this limitation would be overcome in microscale devices.

Direct interaction between bacterial communities, as in a biofilm, may also be preferable in applications such as bioremediation and biorefineries. Moreover, it represents a means for attaching and retaining cell populations in a device. Hong and coworkers developed controllable biofilms consisting of both colonizer and disperser cells [58]. In a microfluidic device, they constructed an initial colonizer population, then introduced a second disperser population, and engineered a robust dual-species biofilm. Using an external signal, the disperser population then removed the colonizer cells, and a chemical inducer could be used to remove the disperser cells. The directed assembly and disassembly of biofilms represents a powerful tool to perform synthetic biocatalysis using genetically-programmed pathway design.

Finally, for a bioproduction breadboard to be fully functional, the placement of its cell types onto or within the device should be rapid, simple, enable interconnectivity as well as connectivity to primary actuating signals. We have developed several strategies that build on stimuli-responsive polysaccharides and proteins for *in situ* assembly of cell populations [59-62] onto devices that preserves their biological functionality and also enables interrogation via optical and electrical means.

Conclusions

We have highlighted advances at the interface of cell signaling, genetic regulation, directed use of microbial consortia, as well as touched on their assembly in device systems that suggest development of a customizable and potentially standardized ‘bioproduction breadboard’. With the rapid advancements in systems and synthetic biology, we are not limited to pathway

engineering among monoculture or even polyculture cell populations. Even synthetic cells could contain artificial circuits with useful functionalities that cannot be translated or ‘misread’ by a natural organism [63]. We note that incorporating electrical signals, as noted earlier, would greatly increase speed and enhance compatibility with electronic devices. Finally, by the incorporation of autonomous dynamic gene expression, the biological elements could self-regulate and optimize as the desired pathway is adjusted, easing the burden on breadboard controllers. The ‘plug and play’ nature of these systems could allow for accelerated screening of novel metabolic pathways, increased functionality through rapidly changeable networks, and unprecedented sophistication as diverse cell populations are linked by function in pathway designs.

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Figure 1: An example of the interchangeable connections of the 'bioproduction breadboard'. Lignocellulose can be degraded by *T. reesei* into simple sugars. In one pathway, the sugars could be inputted into a strain of *E. coli* to produce butanol. Alternatively, the flow into *E. coli* could be shut off and the sugars could be fed to *S. cerevisiae* to produce free fatty acids.

Figure 2: Electrical assembly and control of enzymatic biosynthesis. Gordonov et al [21] developed enzymatic assembly and control using electrical inputs. Increased electrical input is provided to the electrode (left to right) which results in decreased enzymatic activity (left to right).

Figure 3: Dynamic gene expression. **A)** Autonomous pathway redirection is depicted where metabolic pathways that are directed towards cell growth (solid gray lines) are shunted towards product synthesis (dashed grey lines) when cell density reaches a sufficient threshold **B)** Autonomous pathway balancing is depicted as metabolic pathways towards product synthesis are continuously balanced through upregulation and downregulation of intermediates to maintain optimum flux balance.

Unicellular

Consortia

Figure 4: Synthetic consortia. A schematics of the benefit of specialization in consortia are depicted. Unicellular organisms are inputted with the full metabolic pathway, and this higher workload results in reduced metabolic flux to generate product. Multicellular systems have specialized production responsibilities, resulting in increased metabolic flux.

Graphical abstract