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Cybergenetics: Theory and Methods for Genetic Control Systems

M. Khammash, M. Di Bernardo, and D. Di Bernardo

Abstract— **This tutorial article gives an overview of the theory and design tools for the real-time control of living cells. The theoretical, computational, and experimental tools and technologies utilized for achieving such control make up a new and exciting area of study at the interface between control theory and synthetic biology, to which we refer as *Cybergenetics*. This article (along with [1]) accompany a tutorial session on Cybergenetics, that is intended to introduce control scientists and engineers to the different ways living cells can be controlled, and to the many opportunities for future developments—both theoretical and practical—-that such control brings about.**

I. INTRODUCTION

Norbert Wiener's celebrated 1948 book *Cybernetics*, presented a remarkable vision in which he unified control and communication studies in the animal and the machine. Predating the discovery of the structure of DNA and the ensuing molecular-biology revolution, contemporary applications of cybernetic ideas in the life sciences were limited. More than six decades on, the confluence of modern genetic-engineering techniques, new measurement technologies, and a deeper understanding of cellular processes is enabling for the first time the emergence of a cybernetics at the *molecular scale*—one in which novel control and estimation concepts are developed and used for precisely and robustly regulating cellular processes using *genetic* methods. This concept along with supporting theory and design tools is referred to as **Cyber·genetics**. Lying at the interface of control engineering and synthetic biology, the implications of Cybergenetics are far reaching, with substantial potential impact in basic science, biotechnology, and medical therapy. At the same time, cybergenetics will open up a new direction in the field of control and estimation theory.

Enabling Technologies: Genetic engineering has revolutionised biological sciences, biomedicine and biotechnology. In biotechnology, applications include protein production, such as replacement hormones, enzymes, or vaccines, while in therapeutics applications have focused on diagnosis and gene therapy to treat genetically based diseases [2]. Genetic engineering technology not only facilitated the manipulation of individual genes, but it also has ushered in the new era of synthetic biology [3], [4], where several new gene arrangements with interacting protein products can be engineered into living cells. This makes possible the synthetic construction of new genetic circuits with improved or entirely new function. The promise of the field is that circuits thus built can be composed into devices and systems [5], with unlimited expansion potential. Already circuits implementing oscillators [6], [7], toggle switches [8], and logic gates [9] have been designed and successfully tested in living cells.

The Promise of Cybergenetics: The ability to robustly steer cellular behavior in a prescribed fashion will be transformative, enabling myriad applications in biotechnology, chemical industry, health and biomedicine, food and the environment. For example by employing control systems to manipulate enzyme concentrations in a prescribed manner, one can precisely control and enhance bio-product formation while keeping toxic byproduct formation in check. This can be achieved in spite of poor process models, disturbances, and cell-to-cell variability. Even small improvements can have a big impact in the development of drugs, vaccines, antibiotics, antibodies, etc. Another very promising area of application is synthetic device therapy. Many human diseases are characterised by the failure of regulatory mechanisms. Genetically engineered control systems that dynamically sense dysregulated physiological variables and respond by producing appropriate biological effectors (e.g. hormones) can return these variables to tightly controlled ranges, thereby reinstating proper regulation and providing a viable path to treatment. The theory and methodology needed to enable the rational and systematic design of cybergenetic systems will not only facilitate countless applications with far reaching impact, they will also open up new directions of research in the fields of control theory and estimation theory.

II. IN VIVO GENETIC CONTROLLERS

The tools of genetic engineering has made it possible to construct and integrate within the cell controllers that comprise biological molecules which act together to realize real time control. In this regard, these synthetic engineered controllers are similar to the endogenous control systems that cells have naturally evolved. We shall refer to these controllers as in vivo (or embedded) controllers (Fig. 1B).

Next we describe some of the key synthetic in vivo controller implementations in living cells; other controllers are also described in [10] and [11].

A. Genetic Controller Implementations

In the early days of synthetic biology, an example of what is possible was presented in [12] where a simple negative

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M. Khammash is with Faculty of Biosystems Science and Engineering, ETH Zurich, Switzerland mustafa.khammash@bsse.ethz.ch

M. Di Bernardo is with the Faculty of Engineering, University of Bristol, UK m.dibernardo@bristol.ac.uk

D. Di Bernardo is with the Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy dibernardo@tigem.it

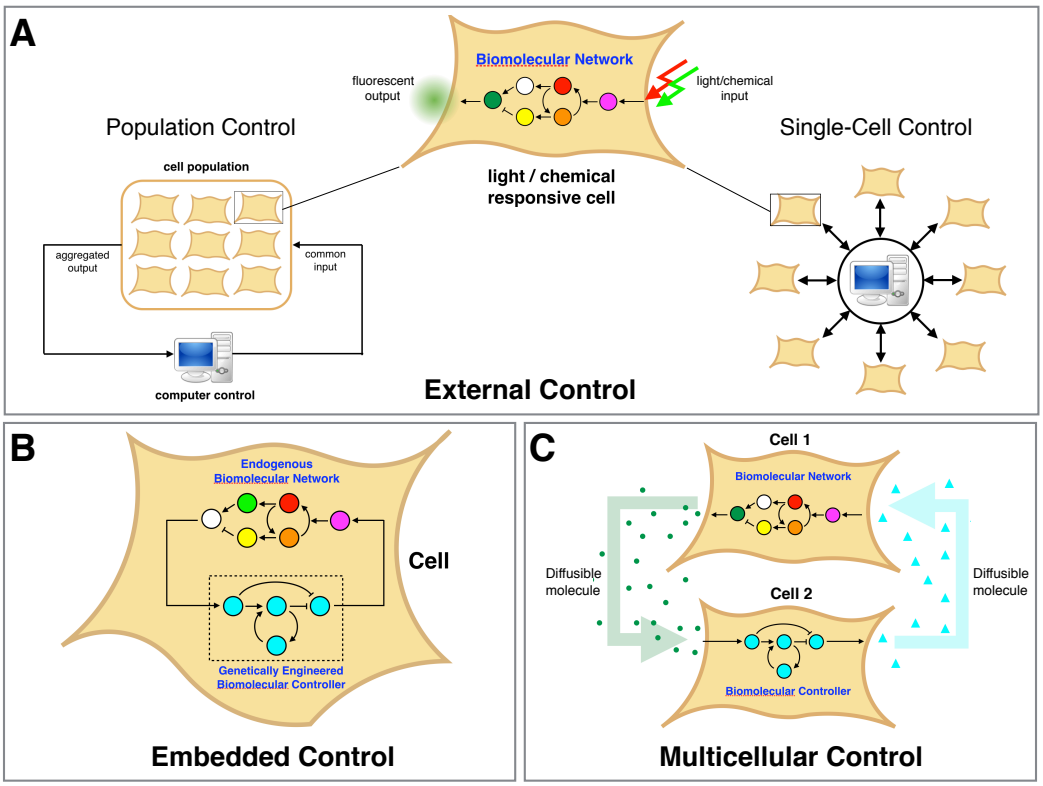

Fig. 1. Cybergenetic control modalities. **Panel A** shows how external (also referred to as in-silico) control is implemented. Cells are engineered to respond to light of a particular wavelength or to certain chemical inducers. The output is typically a fluorescent protein whose abundance is reflected in the fluorescence intensity which can be measured either with a microscope or a flow cytometer. The controller is implemented on a digital computer interfaced with the living cell(s) to close the feedback loop. External control can be applied to control cell populations or single cells independently. In the former, an entire cell population is controlled with a single input, usually either light or a chemical inducer whose concentration can be controlled. The output is the averaged output of the population (e.g. average protein fluorescence). In the latter approach, single cells grown in monolayers are controlled individually and independently with a separate controller regulating each cell. This is usually carried out using light, which targets individual cells using an array of micromirrors that can be precisely controlled so that each cell is exposed to the desired intensity as prescribed by its own controller's command input. **Panel B** shows how an in vivo (also referred to as embedded controller) is implemented. Here the controller components are themselves biomolecular species that are synthesized by the cell's machinery. Their biochemical reactions among these species define the dynamics of the controller. Such species also interact with the biochemical species that define the plant through biochemical reactions. The collection of plant and controller molecular species and their biochemical interactions make up the closed-loop system. **Panel C** shows how a multicellular control system works. Like the in vivo (or embedded) controller, the controller species consist of biochemical species. However, here the controller species and the plant species are contained in different cells. Interactions between the plant and controller cells happens through diffusible molecules.

feedback gene circuit was constructed in *E. coli* and demonstrated to give stable regulation and reduced variability—key attributes of negative feedback. Other implementations of simple genetic control circuits followed. In [13] synthetic positive- and negative-feedback loops were constructed by dynamically regulating recruitment of pathway modulators to an artificial binding site in yeast cells. These engineered circuits yielded diverse behaviors such as ultrasensitive (high-gain) steady-state response, accelerated or delayed response times, and tunable adaptation. In [14] the authors used small non-coding RNAs, called micro RNAs, to implement positive and negative feedback loops that attenuate fluctuations in protein levels in mammalian cells.

In [15] synthetic protein scaffolds were used to implement concentration tracking through negative feedback. The proposed biomolecular concentration tracker circuit was shown to achieve dynamic protein concentration tracking in *E. coli*. In the context of mammalian cells, the work reported in [16] described a platform that combined a ligand-responsive ribozyme switch and synthetic micro RNA regulators to create a control device based on RNA interference (RNAi). This was then utilized to build a negative feedback control

system that acted as a proportional controller that functioned to maintain target intracellular protein levels in response to increases in transcription rate. Integral control implementations in living cells posed a particular challenge. The first synthetic integral feedback controller in living cells was reported in [17] (see also [18]) where a certain biochemical reaction motif, called antithetic feedback, was used to realize a practical implementation of integral control in *E. coli* and to demonstrate the perfect rejection of constant disturbances. A quasi-integral feedback controller implemented in *E. coli* using small RNA was subsequently reported in [19], while an in vitro (cell-free) implementation was recently reported in [20]. The theory and implementation of integral feedback will be discussed in more detail in the next section.

Over the last year or so, progress in synthetic controller implementations has accelerated. In [21] the authors modelled, built and validated two synthetic negative feedback circuits that use rationally-designed small RNAs in *E. coli*. In one circuit an externally-inducible sRNA was used to tune the effective feedback strength, while in the other the output is a transcription factor that induces expression of an sRNA, which inhibits translation of the mRNA encoding the output resulting in a negative feedback loop. In mammalian cells, micro-RNAs can be effective tools for implementing regulation. In [22], a novel family of gene expression control systems of varying complexity and enhanced performance were proposed. These include an incoherent feedforward circuit that exhibits output tunability and robustness to plasmid take-up variation, a negative feedback circuit that reduces burden and provides robustness to transactivator dosage variability, and a new hybrid circuit integrating negative feedback and incoherent feedforward that combines the benefits of both. The benefits of these control circuits were demonstrated in human-induced pluripotent stem cells where they were shown to enable precisely regulated expression of an otherwise poorly tolerated gene of interest. The expression of proteins from synthetic circuits introduces additional burden that cells are not adapted for. The authors in [23] devised a dCas9-based feedback-regulation system that automatically adjusts the expression of a synthetic construct in response to burden. Cells equipped with this controller maintained their capacity for native gene expression to ensure robust growth and thus outperformed unregulated cells.

B. Integral Feedback Controllers: Theory and Implementation

In this section, we focus on the molecular realization and implementation of integral feedback as as an example of in vivo control. It has been well established in control theory that integral feedback is necessary and sufficient

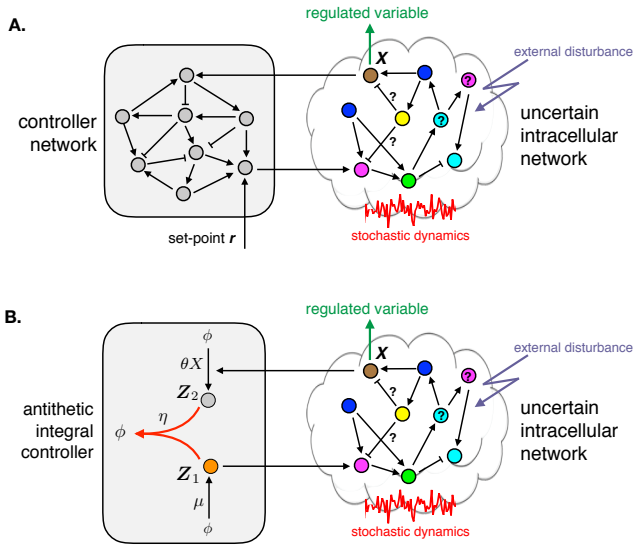

Fig. 2. Robust perfect adaptation and integral control. **Panel A.** shows the setup of the robust perfect adaptation control problem. Given a stochastic dynamical systems (plant), the goal is to find a set of chemical reaction networks (controller), such that the interconnection of plant and controller achieves stability and drives an output of interest X to the setpoint r asymptotically in spite of uncertainty in the plant model and parameters and in spite of constant external disturbances. **Panel B** shows one solution to the robust perfect adaptation control problem. The controller implements a biomolecular integral feedback control system that functions in the presence of noise. The integral controller is realized through interactions of two species and 4 reactions configured in an arrangement referred to as antithetic feedback motif .

for robust steady-state tracking and disturbance rejection of constant inputs for linear systems. In biology this property is commonly referred to as *robust perfect adaptation* (RPA), because the disturbance is thought of as a stimulus that must be adapted to. In spite of the discovery of integral feedback in natural biological systems (see e.g. [24], [25], [26], [27]), its synthetic implementation in living cells has been challenging. The nature of the challenges inherent in the implementation of a genetic integral controller were outlined in [28].

The problem of robust perfect adaptation in the biomolecular setting can be posed as follows. Given a set of intracellular biochemical reaction networks shown in Fig. 2A (considered here to be the plant to be controlled). We assume that this intracellular network is uncertain, either because some of its reacting species are unknown, or because of the reactions are unknown (or both). We also assume that the network has stochastic dynamics due to the fact that at the molecular level the timing and order of chemical reactions exhibit randomness. The dynamics of such system are governed by stochastic chemical kinetics, see e.g. [29], which can be described mathematically as continuous-time

discrete-state Markov processes. This uncertain stochastic plant may be subjected to constant external disturbances. We also assume that the concentration of one of the species, X, is to be steered to a desired reference value r in the steady-state. The control problem of interest is to design an set of stochastic chemical reactions (controller network) such that when augmented with the intracellular reaction network, the resulting interconnection (closed-loop network) achieves ergodicity (a notion of stochastic stability that ensures the network has a unique stationary distribution) and ensures that $\mathbb{E}(X(t)) \to r$ as $t \to \infty$ (robust perfect adaptation). A solution to this problem was given in 2016 by Briat et al. [30], where it was shown that under reasonable conditions on the ergodicity of the interconnected network, a particular chemical reaction network motif, called antithetic feedback, achieves the exactly the desired objectives. This motif consists of two controller species Z_1 and Z_2 and four reactions as follows:

$$\phi \xrightarrow{\mu} \mathbf{Z}_1; \quad \phi \xrightarrow{\theta \mathbf{X}} \mathbf{Z}_2; \qquad \mathbf{Z}_1 + \mathbf{Z}_2 \xrightarrow{\eta} \phi; \qquad \phi \xrightarrow{k \mathbf{Z}_1} \mathbf{X}_a,$$

where $\mathbf{X}_a$ is the actuated species (plant input). It was shown that such a controller implements integral feedback such that

$$\mathbb{E}(Z_1(t) - Z_2(t)) = \int_0^t \mu/\theta - \mathbb{E}(X(s))ds,$$

and therefore ensures that robust perfect adaptation is achieved, i.e. that

$$\mathbb{E}(X(t)) \to \mu/\theta \text{ as } t \to \infty.$$

Note that the steady state value of the output does not depend on the plant parameters and only depends on the setpoint μ/θ.

The practical implementation of such an integral controller requires two species Z_1 and Z_2 that annihilate each other, or mutually inactivate each other through sequestration or other means. Such an implementation was first achieved in [17], [18] where a tightly binding sigma/anti-sigma protein pair served as realizations of the controller species Z_1 and Z_2. In this work, tunability of the set point as well as robust perfect adaptation to a constant disturbance were demonstrated experimentally in *E. coli* cells. An application of this integral controller to robustly regulate bacterial growth in the face of large temperature shifts demonstrated the practical applicability and promise of integral control in genetic circuits. On the theoretical level, the work in [18] mathematically proved that the antithetic controller motif is universal, in the sense that it is both necessary and sufficient for robust perfect adaptation for systems with noisy dynamics. It further presented simple nonconservative algebraic conditions for a controller to achieve robust perfect adaptation, providing a parametrization of all controllers that achieve robust perfect adaptation for systems with noisy dynamics.

Performance tradeoffs for antithetic controllers were studied in [31] who focused on the stochastic case, and in [32], [32] who studied the problem in detail in the deterministic setting and provided insights on the fundamental tradeoffs and hard limits on performance. An alternative integral feedback design in the deterministic case was reported in [33]. Other work related to the antithetic motif was also given in [34].

C. Challenges for in vivo control

In spite of the above developments, and the availability of more powerful genetic tools, the implementation of more sophisticated controller designs requires that several practical and conceptual problems be addressed. A living cell consists of networks of dynamic interactions among DNA, proteins and metabolites that achieve cellular functions such as signaling, stress response, material and energy transfer, growth. To fully realize the promise of cybergenetics, control at the cellular level must contend with several challenges peculiar to the cellular environment and the molecular basis of the dynamics within. The environment in the living cell is unlike that in which traditional engineering control systems operate, requiring the development of new control methodology customized to that environment. Some of the main challenges facing in vivo control system design are as follows.

Intrinsic stochastic noise. Unlike in a test tube, chemistry inside a living cell is inherently stochastic [35]. At the subcellular scale, the timing and order of chemical reactions is random, and reacting species (e.g., DNA, mRNA, proteins) are often present in low abundance (e.g., ones to tens of molecules). This intrinsic randomness cannot be masked by the law of large numbers, and its effects are measurable and consequential. Intrinsic noise manifests as fluctuations in species abundances over time, and is a main source of cell-to-cell variability [36].

Context dependence. Further key contributors to cell-to-cell variability are differences in the specific context for each cell, including the local microenvironment, resource abundances, gene dosage and cell-cycle stage [37]. Whereas intrinsic noise leads to stochastic dynamics, context dependence means that the parameters for these dynamics may be different among cells.

Unknown or uncertain network topologies. Our understanding of cellular networks has been improving dramatically, but key molecular players and interactions remain

missing. As a result, models of cellular dynamics are uncertain and incomplete. This sort of uncertainty is also common in engineering systems, but the scale is larger in cellular systems, owing to their complexity and the frequent interaction among their subsystems. As with traditional control applications, this uncertainty makes feedback control essential for robust regulation [38].

Metabolic burden. When control systems are genetically engineered into living cells, their synthesis and maintenance by the cell could pose a metabolic burden that adds to the metabolic load of the cell [39]. This is potentially a problem for smaller fast-growing cells, such as bacteria. If not taken into consideration, this metabolic load could lead to the elimination of the controlled cell in the population, as random mutations that result in controller disruption will lead to faster-growing cells that outcompete the controlled ones.

Latency of controlled variables. Whereas direct measurement of the controlled cellular variable(s) is most desirable, oftentimes only indirect measurements are possible. For example, the measurement of a protein of interest might be difficult, and only measurements of a downstream protein may be practical. Dynamic estimation of the controlled protein from the downstream protein measurements can provide an effective alternative [40]. An estimation theory that respects the molecular nature of cellular dynamics would be highly enabling.

III. EXTERNAL CONTROL OF LIVING CELLS

External controllers are implemented as a computer software and interfaced with living cells by means of microfluidics to grow cells, light emitting diodes or automated pumps to provide inputs, and a microscope to measure the output, as shown in Fig. 1A. Microfluidics devices can be mounted on standard microscope slides and have features such as channels to provide reagents to the cells, and chambers to host the cells, whose dimensions are in the order of micrometers. At this scale, fluid physics dictates that flow regime is laminar hence no turbulence can occur. Moreover, several principles of classical electrical engineering, such as Ohm's second low, can be applied by substituting voltage differences with pressure differences and currents with fluid flows. Microfluidics is an experimental technology that allows: (i) to limit the operating costs associated to experiments, because of very small volumes of reagents needed (a few microliters); (ii) to rapidly change the micro-environment in which cells are growing, and (iii) to obtain real-time in-vivo quantification of reporter proteins (outputs), as the cells can be continuously grown in the device under the microscope. Microfluidics devices can be tailored to specific applications and organisms (bacteria, yeast or mammalian cells) and have been successfully used to implement external controllers [41], [42], [43], [44], [45], [46], [47], [48], [49]. These controllers can be distinguished in two broad categories depending on the nature of the control input: (i) optogenetics-based inputs where light at a specific wavelength is used to control specific processes such as transcription of a gene. Optogenetics has the advantage that each cell in a population can be controlled individually, thanks to projectors using Digital Mirror Devices able to shine patterned light onto multiple cells at the same time thorough the microscope objective; (ii) microfludics-based inputs, where the same chemical or physical stimulus is applied to all the cells at the same time by means of a change in the micro-environment within the cell chamber via the microfluidics channels. Microfluidics-based inputs have the advantage of being simple to implement and that the control input can be biologically relevant (a drug, a hormone, etc.), however each cell will receive exactly the same control input.

A. External controllers with microfluidics-based input

An external controller implemented with a microfluidics-based input performs the following steps: (1) the output of interest is measured in cells. This is usually a fluorescent protein whose level is observed with a time-lapse fluorescence microscope taking images at given sampling time (in the order of minutes); (2) protein level is quantified in individual cells from fluorescent images with a custom image processing algorithm; (3) a computer implementing the control strategy computes the control input needed to minimise the difference between the target level and the actual population-averaged fluorescence intensity across the cells; and (4) an automated set of syringes or pumps delivers the appropriate amount of molecule to the cell chamber in the microfluidic device, thus closing the loop.

One of the first examples of external control with a microfluidics-based input is reported in Uhlendorf et al. [50] to control the expression of a reporter protein from the Hog1-responsive promoter in yeast cells by using changes in osmotic pressure as control input. Authors demonstrated that a Model Predictive Control (MPC) strategy was effective in achieving both constant and varying amount of fluorescence at the population and even at the single cell levels.

In a series of studies [48], [51], the use of two different sugars (galactose versus glucose) to control in real-time gene expression in yeast cells from the GAL1 inducible promoter was presented. The authors demonstrated the ability of the platform to satisfy set-point and tracking objectives when controlling gene expression either with Proportional−Integral controllers or MPC controllers, where the control inputs were galactose and glucose, while the

output was the average fluorescence level of a reporter protein downstream of the GAL1 promoter across yeast cells. More recently, this strategy was used to precisely control the levels of the human $\alpha-$synuclein protein in yeast cells engineered to express the protein from the GAL1 promoter [41]. $\alpha-$Synuclein is known to form aggregates in human neurons of Parkinson's Disease patients, and aggregation is known to be concentration dependent. By controlling the amount of $\alpha-$synuclein protein at different levels in yeast cells, the authors were able to show that formation of $\alpha-$synuclein inclusions is strictly concentration, but not time, dependent and that the threshold needed to form inclusions for the wild-type $\alpha-$synuclein form is about the double of the mutant form, which causes early onset PD [41].

Recently, external control was applied to a bistable nonlinear genetic circuit, the toggle-switch in bacterial cells, that exhibits two stable states resulting from two proteins mutually repressing their own production [45]. The amount of repression exerted by the two proteins on each other can be differentially modulated by providing two molecules. The control objective was to stabilise the unstable equilibrium point, where the repression forces exerted by the two proteins on each other are comparable. The authors show that open-loop control with a control input consisting of periodic forcing alternating between the two molecules is able to maintain the majority of cells close to the unstable equilibrium point. An interesting theoretical analysis of this controller has been recently reported [52], [53].

Finally, it is worth noting that external controllers with microfluidics-based inputs have been successfully applied also to mammalian cells [43]. Specifically, an inducer molecule was used to automatically regulate gene expression from inducible promoters in different cell types, including mouse embryonic stem cells, using as control input the concentration of an antibiotic, as well as to precisely regulate the activity of the mTOR signaling pathway in single cells, using as control input the concentration of a small molecule inhibitor of the mTORC1 kinase [43].

B. External controllers with light-based (optogenetic) input

One alternative to using small molecule chemical inducers as inputs in microfluidics chambers is to use light. To achieve this, the cells must be genetically modified to respond to light inputs. Many such light responsive systems have been developed and engineered into living cells, as part of a growing field called optogenetics. As an inducer, light offers unique advantages, facilitating fast, targeted, low-cost, and precise spatiotemporal modulation of protein function with low-to-no toxicity, while avoiding the pleiotropic effects of small-molecule inducers. Furthermore, promising biotechnological applications such as the control of metabolic activity in microbial production strains require the use of large-volume liquid cell cultures, which cannot be achieved with microfluidics.

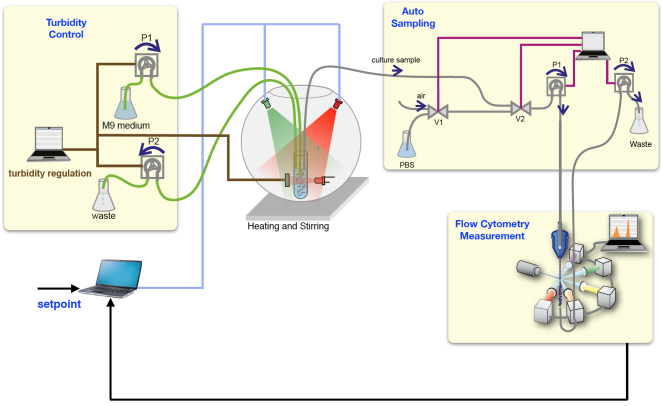

Fig. 3. External feedback control loop using light input. The setup shown is typical of optogenetic feedback control loops. Cells are grown at a constant density using a turbidostat which employs a simple control loop. Cells are regularly sampled using an autosampler consisting of a system of valves and pumps. Samples are fed to a flow cytometer, which measures the distribution of the expressed proteins in the cell population. The average value of such proteins is the regulated variable, which is fed to the control computer. The computer implements the control algorithm (a proportional-integral controller or a Model Predictive Controller). The controller output is the light intensity which activates gene expression in the living cells, thereby closing the feedback loop.

A major area of application of light control involves the control of gene expression. Optogenetic gene expression control can be achieved in an open-loop fashion. Such control, however, requires finely tuned mathematical models that are obtained in lengthy characterization processes and inherently lacks robustness, limiting the general applicability of the approach in biotechnology. Closed-loop optogenetic control of gene expression was pioneered in 2011 in [54] where it was shown how cells can be interfaced in feedback with digital computers using light as a communication medium. In this work, a Phytochrome B/Pif3 red/far-red light system was used to precisely and robustly control gene expression in yeast cells. The controller, which was implemented on a digital computer, used Kalman filtering and Model Predictive Control (MPC). More recently, optogenetic feedback was used to control gene expression in bacteria [47]. The control setup used in this work is shown in Fig. 3. This research demonstrated the effectiveness of *in silico* optogenetic control in achieving precision and robustness to day-to-day variability in cellular behavior, to changes in the environment (temperature, media, etc.), and to large model uncertainties. It also showed how optogenetic feedback control can be used to

precisely control bacterial population growth rates to desired set points. This work presented a proof-of-concept to the idea that closed-loop light control can be used in bioreactors to tightly regulate key biological processes that can in turn optimize bio-production.

Due to its finely controllably spatiotemporal patterns, light can also be used to control single cells or even cellular substructures. In [42], a digital micro-mirror device consisting of millions of independently controlled MEMS mirrors was developed and used to simultaneously and independently control stochastic gene transcription in hundreds of yeast cells in a closed-loop fashion. Gene expression of individual *E. coli* cells was similarly controlled by light in [55].

IV. MULTICELLULAR FEEDBACK CONTROL

In vivo (or embedded) genetic controllers can sometimes be cumbersome to integrate in a single cell. Indeed potential metabolic burden can be self defeating for the host [56], [57]. Also once cells have been synthetically engineered, any change in the control strategy or its application requires re-engineering the entire control system leading to poor modularity and adaptability of the original design and its parts [58]. To overcome these limitations an alternative approach to those described so far is to adopt a *multicellular control* strategy where the various functions needed to implement a control system are distributed across different cell populations within a microbial consortium.

Microbial consortia have been highlighted as a solution to achieve highly modular, scalable and more robust design, as for example discussed in [59], [60]. Indeed the interaction of microbial populations can be advantageous in accomplishing complicated tasks better than a single population can do, whilst beneficially guaranteeing increased robustness to environmental fluctuations [61], [62]. Moreover, the engineering of synthetic microbial consortia makes it possible to physically separate the components and modules required to achieve the desired functions, hence reducing the unwanted effects of retroactivity in biological circuits (as defined in [63]) by which standard parts available in Synthetic Biology can significantly change their behavior upon interconnection [64].

In the multicellular control approach one cell population (the "controllers") senses and regulates some phenotype of another population (the "targets"). Specifically, as depicted schematically in Fig. 4(a), the controllers receive an external signal (e.g., an inducer molecule) so that the desired reference level of the process to be regulated in the target cells can be set. In order to establish the control loop the two populations need to communicate with each other so that the control input, $\hat{u}$, can be sent from the controllers to the targets, and the process output, $\hat{y}$, can be fed back from the targets to the controllers. Also the controllers must be able to sense the target outputs, compare it with some reference signal of interest and vary their output accordingly.

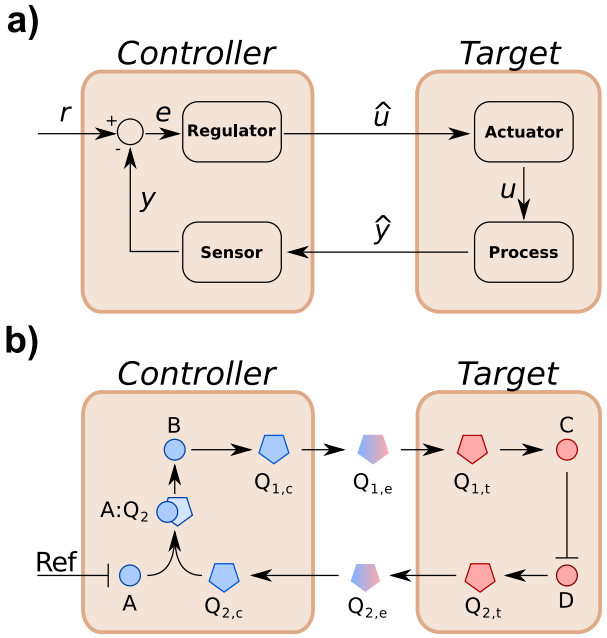

Fig. 4. Main idea (a) and schematic biological implementation (b) of multicellular control: the sensing, actuation and computation functions are distributed across two different cell populations, the controllers and the targets communicating with each other via orthogonal quorum sensing channels (reproduced from [48]).

To achieve this goal appropriate orthogonal quorum sensing channels to implement cellular communication among the two populations need to be selected and a reference comparator module needs to be implemented. A possible schematic biological implementation of a multicellular control strategy was first proposed and analysed in [48] and is reproduced in Fig. 4(b). Here, an external reference signal ("Ref") enters the controllers inhibiting production of a species, say A, that in complex with the quorum sensing molecule Q_2 coming from the targets generates another species B which, in turn, catalyzes the synthesis of another signaling molecule, Q_1. Such a molecule is then released from the controllers into the growth medium and received as an inout by the targets. The control loop is then closed through a species D which catalyzes the synthesis of the sensing molecule Q_2, whose concentration in the growth medium is interpreted by the controllers as a proxy of the system output.

The approach was validated in-silico and shown to be effective and robust to relatively large parameter variations [48], [65]. A comparator module was also implemented in-vivo in [66] where a molecular titration system composed of an orthogonal σ and anti-σ pair was used to compute the relative amounts of the quorum sensing molecule HSL and the chemical activation IPTG, and tune the expression of a Green Fluorescent Protein (GFP) proportionally to the excess of HSL over IPTG. Extensive agent-based simulations reported in [48], [65] showed that a crucial aspect for in-vivo implementation is the ratio between the two populations that needs to be maintained within an acceptable range in order for control to be achieved. Also, orthogonality of the quorum sensing systems is essential to avoid cross-talk among the populations that can affect the quality of the control performance. With this respect synthetic quorum sensing systems might be benefical to implement communication across the two populations within the consortium [67], [68], [69] as well as the analysis recently reported in [70].

Multicellular control strategies can be also essential to enable cellular functions over space. For example to induce spatial patterns to cells that could be useful is several applications such as those requiring coordinated self-organization of cells [71], [10]. Also, control strategies across multiple populations have been reported as useful to control cell growth and maintain populations' size at a desired equilibrium [72]. For example, in [73] a population control circuit is proposed in-silico to autonomously maintain the density of E. coli at a desired level, a goal also achieved more recently in [74] where feedback control across two populations is used for controlling the population size of a microbial culture through the production of toxins and anti-toxins between the populations.

While having been tested in a number of scenarios in-silico, multicellular control has not been fully implemented in-vivo yet. Also, in-silico validation often relies on the use of aggregate deterministic models or agent-based simulations. Hence, the full experimental implementation and validation of a multicellular control strategy remains a crucial open problem as well as its modelling via approaches that can fully take into account the unavoidable stochastic and spatial distribution effects it entails. Also, another important open challenge is the study and implementation of multicellular control strategies for other species beyond *E. coli* as for example for mammalian cells where applications of multicellular control can be even more relevant, as for instance to the area of regenerative medicine or the development of personalized therapies.

V. BIOCONTROL EXPERIMENT: STARTING A LAB

This section aims at giving a broad overview of the key steps needed to set up a lab to perform biocontrol experiments using microfluidics with microfluidics-based inputs and an inverted microscope. Alternative approaches are possible, such as the use of plate readers to measure the control output, or cytofluorimetry. The idea of setting up an experimental lab from scratch can be daunting, especially if the expertises required are cross-disciplinary as the case of Control Engineering and Molecular and Cell Biology. However, with a little effort and some perseverance, this can be done in a less than a year, even with limited funding and it can be extremely rewarding. Rather than giving a list of parts needed, this section is meant to provide a set of general guidelines and useful suggestions to avoid common mistakes. The first suggestion is to start small focusing on bacteria or yeast rather than mammalian cells, this will reduce costs, time, equipment and training needed. The second suggestion is to ask for guidance to colleagues in the Biology Department, a few days of training in a standard lab will spare you a lot of time and frustration. Also these colleagues will be essential to speed up delivery of necessary products, as they already know whom to order from. The third suggestion is to use microorganisms already engineered and ready to use, rather than engineering your own. This can be easily achieved by writing to the corresponding author of a manuscript describing the engineered microorganism of interest and asking for it, usually the microorganism will be delivered to your lab in a few weeks free of charge (except for the mailing costs). The workhorse of the lab is the bacterium *E. coli* and most of the Control Engineering applications have been performed in this microorganism. *E. coli* grows very rapidly with a doubling time of about 20 min, which means that overnight cultures usually provide enough material to perform experiments. Working with *E. coli* presents virtually no risk so they can be grown on a bench but must be kept at 37 °C and, when grown in liquid culture, agitation is preferred, so you will need to buy or build yourself a shaker. Growth medium is quite cheap and bacteria can be stored in standard fridge for a few months (+4 °C and -20 °C); long term storage requires -80 °C and such fridges are bulky and expensive so the best is to ask for some space from colleagues. Antibiotics are also needed to avoid contamination. Of course, you will also need some disposable plastic-ware such as culture dishes and pipette tips. Similar considerations apply to the yeast *S. cerevisiae* that has also been extensively used in cybergenetics applications.

In order to perform your first biocontrol experiment, you

will need access to an inverted microscope, this is the most expensive equipment with a cost of up to 100,000 euros, however if that kind of funding is not available, or if you are not ready to invest in it, this is not a problem, as most Biology Departments have central microscopy facilities with a fee-for-service open to faculty. Lastly, you will need to set-up microfluidics in the lab. Again, this is quite difficult if you want to do everything on your own without asking for help, on the contrary if you ask a colleague who already has the set-up in place for training, a month will be enough to get the basics and replicate the set-up in your lab. There are two main alternatives when implementing microfluidics technology with the biggest difference being how fluids are provided to the microfluidics chip: (i) pump based methods are the most popular but require pumps and electro-valves, both of which are quite expensive, whereas (ii) an hydrostatic pressure based system proposed by the lab of Jeff Hasty, simply exploits the difference in height between two syringes to drive the flow within the microfludics device [75]. The syringes hang on the wall and are connected via capillary tubes to a Y junction upstream of the cell chamber. The difference in the relative height causes the fluid from one syringe, or the other syringe, or a mix of both to get to the cell chamber. This technology was named Dial-a-Wave and can be implemented in the lab with less than 500 euros. A step-by-step guide to implement the Dial-a-Wave technology is given in http://biodynamics.ucsd.edu/dialawave/. The Dial-a-Wave system has been used to design microfluidics devices for bacteria, yeast and also mammalian cells. Hence, it is better not to design your own device, but to use designs that have been already tested and tried. This has the other advantage of skipping the most laborious and expensive part of microfluidics, that is to design the device.

Microfludics chips can be fabricated in a few days at a cost of less than 5 euros per chip by means of the replica molding technique, once a master mold is available. The master mold is a silicon wafer where by means of photolitography the microfludics device is etched. Usually photosensitive resins, called photoresists, are spun over silicon wafer; a mask with the chip design is put between the wafer and a UV light source. UV light exposed photoresist will polymerise and become resistant to a developer solution. This reagent will eliminate all uncross-linked photoresist molecules leaving on the wafer a precise profile of the desired structures. Once the master is available the replica-molding protocol can be used to obtain a large number of identical devices. The fabrication of a microfluidics device starts with the pouring of the Polydymethilsiloxane (PDMS) liquid pre-polymer and curing agent mix on the master mold. PDMS is very cheap (200 euro/kg) and can be easily purchased. The petri dish containing the polymer is then baked at 80C for 2 hours and then the PDMS is cut and peeled off from the master. The new devices can then be irreversibly bonded to a microscopy glass slides by reactive oxygen treatment of both the polymer pieces and slides. For this you will need a plasma surface preparation machine, but a standard microwave oven microwave can also be used [76]. Alternatively, reversible bonding can be achieved, for example by applying vacuum, if a vacuum source is available next to the microscope. At the end of this step the device are ready for inspection for anomalies and defects. The key step for microfludics chip fabrication is the silicon master mold, which if properly handled can last for several years and used to generate thousands of chips. The easiest way to obtain a master mold is to ask a colleague using the same microfludics device how she/he obtained it. Alternatively, starting from the CAD file with the microfluidics design, commercial companies are available (www.micruxfluidic.com) but depending on the features of the devices prices may vary.

With the Dial-a-wave microfluidics system in place, you are ready to perform your first biocontrol experiment, but you need some additional software to enable real-time feedback control, including image segmentation algorithms and software to control the microscope. Again the best advice is to start by asking a colleague who has implemented biocontrol in her/his lab for help. In any case, several open-source pieces of software have been developed to help with this task. To interface the microscope with the computer, the open-source microscopy software 'Micro-Manager' is the place to start (https://micro-manager.org/), whereas for image segmentation CellStar is an easy to use and versitile tool (https://www.cellstar-algorithm.org/)[77].

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