Engineering Principles for Synthetic Biology

from concepts to practice

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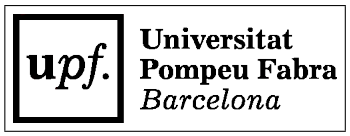

Hay hombres que luchan un día y son buenos. Hay otros que luchan un año y son mejores. Hay quienes luchan muchos años, y son muy buenos. Pero hay los que luchan toda la vida, esos son los imprescindibles.

Bertold Brecht

A la mare, el pare i l'Ona. *Imprescindibles*.

Agraïments

Aquestes paraules que llegiu són les últimes que escric de la tesi, el colofó final a una obra que és només la punta de l'iceberg d'una etapa molt intensa en molts sentits diferents. De ben segur que les paraules que seguiran no podran fer justícia al que tot plegat ha significat per a mi, a tots els nivells, però espero que s'hi apropin. Finalment, només em queda afegir que l'ordre que segueix el text és per motius únicament literaris i no manté cap relació amb la rellevància que poden tenir les persones que hi apareixen.

Fa poc llegia l'últim llibre del neuròleg Oliver Sacks, on aporta les seves últimes reflexions abans de morir de càncer. Titulat *'Gratitude'*, en aquest llibre l'autor exposa com el sentiment predominant que ell sent en els últims moments, no d'una etapa sinó de la vida mateixa, és el de gratitud. Digueu-me romàntic però m'alegra que les últimes línies que escric per tancar aquesta etapa siguin les que donaran lloc a la secció d'agraïments, on podré expressar la meva gratitud a tots els que m'heu ajudat d'una manera o una altra a que la tesi vegi la llum.

Va ser curiosament a l'edat de 23 anys, just quan començava el meu doctorat, quan vaig passar per una situació vital similar, una que em va apropar perillosament al llindar que separa els morts dels vius. Aquest sentiment de gratitud que Sacks tan bé descriu al seu llibre, molt millor de com jo ho podria expressar amb paraules, és el mateix que em va assaltar aquelles setmanes i que m'acompanya des d'ençà. Afortunadament, i sobretot gràcies al coneixement i a les bones mans dels neurocirurgians i la resta de personal de l'Hospital Clínic de Barcelona, a qui estaré eternament agraït, vaig ser capaç de superar aquells moments tan delicats i vaig començar a obrir-me pas en el camí que ha culminat en aquesta obra que teniu a les vostres mans. Res no podrà pagar el deute que tinc amb ells i elles, però espero que aquest llibre serveixi d'humil homenatge a la seva impagable feina.

De totes maneres, tot i que la seva intervenció va ser crucial, la meva màxima gratitud va dirigida, tal i com es veu en la dedicatòria que obre el llibre, als que Bertold Brecht anomenava els imprescindibles, als que han lluitat i lluitaran tota la vida al meu costat. A la seva ombra més que a cap altre lloc m'avergonyeix no trobar les paraules adequades i precises per expressar el que sento. Ells són els *meus* imprescindibles, el meu far del sud, la intuïció que es concreta en veritat inapel·lable. Hi eren abans, hi són durant i hi seran després.

Pata, Carbo i Ona, sempre agraït, us estimo.

Si està clar que pel que fa a ells parlo més d'un àmbit personal, pel que fa als que són els actors centrals de la meva tesi, amb el meu permís, és clar, m'és molt difícil destriar la part professional de la personal. Carlos y Javier, Javier y Carlos: habéis conformado un binomio que me ha acompañado y ayudado des de los inicios de la tesis y cuya guía ha sido vital para hacer que ésta, y yo mismo, llegáramos a buen puerto. Gracias a los dos por vuestra labor, la mejor valoración o cumplido que puedo hacer de ella es que ha sido un ejemplo a seguir, así como lo sois vosotros. A nivel personal, os agradezco la cercanía que me habéis mostrado siempre y el respeto que me habéis profesado, tanto en el halago como en la crítica. Me habéis abierto la puerta de vuestras casas y en ellas me he sentido siempre a gusto y querido. Gracias. A nivel profesional, de entre todo lo que me habéis enseñado, que es imposible de recoger aquí, me gustaría destacar vuestra capacidad de trabajar en equipo, de potenciar las fortalezas de cada uno y ayudarle a superar las dificultades. Además, aprecio mucho que me hayáis enseñado en qué consiste ésta profesión, y casi añadiría forma de ver la vida, y que lo hayáis hecho de forma crítica, resaltando sus fortalezas pero también mostrándome sus debilidades e inconvenientes. Por todo ello y mucho más, gracias infinitas. Mi amistad es lo mínimo que os puedo ofrecer a cambio, podéis tomarla sin dudar.

Tot seguit m'agradaria mostrar el meu agraïment a tots els membres del Complex Systems Lab amb qui he compartit algun moment de la meva

etapa doctoral, heu estat part molt important de la meva 'família' durant uns anys d'una intensitat molt considerable. Tot i que amb la defensa de la tesi poso fi a la meva etapa al CSL, m'enduc un bagul ple de coses molt valuoses, moltes de les quals gràcies a vosaltres. A tu, Ricard, m'agradaria donar-te les gràcies per obrir-me les portes d'aquest món quan encara era un estudiant àvid de coneixement i experiències. Personalment, espero no deixar de ser aquell estudiant mai. A tu, Salva, company de fatigues i dels *pringadillos* del wetlab, vull agrair-te especialment la paciència d'ensenyar-me al principi com funcionava tot quan anava ben perdut pel lab, així com el consell savi que m'has ofert sempre que te l'he demanat. A ti, Luiño, que entraste al poco de hacerlo yo cuando aún me estaban retocando la circuitería cerebral, te podría agradecer muchas coses que para mí se condensan o ejemplifican satisfactoriamente -se me ha colado un *mente*- en la amistad que hemos forjado. Sin embargo, de todas ellas quiero resaltar la mirada con la que ves y vives el mundo: es especial, sabia e intensa. Poder empaparme un poco de ella me ha hecho sentir un privilegiado. A tu, Eva, a part del típic tòpic de fer-me de mare que se'ls hi acostuma a atorgar a molts tècnics de laboratori, que també, vull agrair-te el carinyo amb el que m'has tractat sempre i l'ajuda, implicació i iniciativa que has tingut amb mi, tant dins com fora del lab. Aina, ha estat un plaer compartir despatx i molt més amb tu i un honor que em suplissis com a joveneta del grup. Gràcies per les converses llargues i interessants, les competicions absurdes, els aprenentatges lingüístics i, sobretot, per les dosis d'humor i bon rotllo. Adriano, a ti te quiero dar las gracias por tus bromas *ad infinitum* y los ratos y conversaciones compartidas a lo largo de estos años, así como por el cariño que siempre -o casi siempre- me has mostrado, es recíproco. Benito, thank you for all the moments we have shared during these years, like your birthday party and many beer-sessions. Once again I want to stress that I was really sorry I could not attend to your Thesis. I Núria, a tu et dono les gràcies per portar-me sempre la contrària, ha estat un plaer divertit discutir amb tu tot i que poc sovint haguem aconseguit arribar a consensos. Pel que fa als postdocs del grup, Sergi, Josep, Raúl i Dani, vull agrair-vos el vostre consell i ajuda quan m'ha calgut, però sobretot el dia a dia carregat d'humor

i experiències de tot tipus, heu contribuït a fer que anar a la feina fos més que un plaer. Val a dir, també, que us les heu empescat perquè mai no hagi notat la diferència d'edat i de *'rang'* acadèmic, sempre m'heu tractat com un igual, i tot i que així és com hauria de ser, sóc conscient que no sempre ho és. Finalment, també vull adreçar-te unes paraules, Bernat. Sempre m'has tractat amb un somriure i carinyo, i tot i que no vam coincidir molt de temps al lab, en guardo un molt bon record. La conversa amb tu ha estat sempre càlida i intel·ligent, lo últim sobretot gràcies a tu. I Amadís, tot i que no ets estrictament CSL, t'hi conto ni que sigui per difusió passiva. Compartir despatx, volley, beer-sessions i moltes risses i confidències amb tu ha estat un regal.

Però al PRBB, per sort, no només hi som els del CSL. És un ecosistema ric, divers i complex i ha estat un plaer i un privilegi formar-ne part. A part de que científicament m'ha aportat moltíssim conèixer gent d'arreu del món i treballs punters en àmbits molt diversos, si em fessin triar em quedaria amb les amistats que he fet. Són el bé més preuat que m'enduc d'aquest lloc. No començaré a posar noms per dues raons: primer, perquè no acabaria, i segon, per no arriscar-me a deixar-me ningú. Vosaltres sabeu qui sou, començant pels de BioEvo i passant per molts altres llocs i persones. Espero sincerament anar-vos retrobant sovint, si no ja m'encarregaré de fer-me pesat.

A més, voldria fer una petita menció a totes les persones que des de la base i fent feines molt diverses fan possible que aquest formiguer segueixi en marxa i que els qui l'habitem ens hi sentim a gust i puguem tirar endavant els nostres somnis. Em ve al cap la Chelo, de la neteja, que em segueix la pista des que vaig entrar a la carrera i amb qui he compartit breus converses i moltes bromes durant tots aquests anys. O la Natàlia, que m'ha ajudat amb tota la burocràcia fins l'últim moment amb la calidesa que li és natural. No puc deixar de mencionar l'Ainara i la Sònia, que ens han servit esmorzars, dinars, cafès i alguna que altra cervesa dia rere dia amb un tracte proper, diligent i molt amable, contribuint a que em sentís com a casa no només quan treballava. Són només alguns exemples però n'hi

ha força més.

També em vull dirigir als meus avis, l'avi Jordi i l'avia Fina. Gràcies per estimar-me com ho feu, per interessar-vos pel que faig tot i que no ho entengueu i per donar-me suport, sempre, en tot allò que faig, com el doctorat. Us estimo, i tot i que no us truqui i us visiti tant com voldríeu, heu de saber que sempre us porto en els meus pensaments i ben a prop del cor. I faig extensives aquestes paraules a la resta de la família, tiets i cosins i més enllà. Esteu pendents de mi, m'animeu sempre. Gràcies de tot cor.

I ara que parlem de família, em toca dirigir-me als meus amics i amigues. Egoistament, tampoc m'arriscaré a donar noms perquè em faria massa mal si me'n deixo algun i en realitat ja sabeu qui sou i com d'importants sou per a mi. No és broma si dic que sense vosaltres això no hauria estat possible. Quan fas un doctorat, desconnectar-ne és vital. Veure que hi ha llum i vida més enllà és imprescindible. I vosaltres ho heu fet a les mil meravelles. Heu aguantat les meves 'chapas' i les meves queixes. M'heu animat en tot moment i us heu interessat pel que feia, des de les formigues a les que xafo el cul fins als bacteris amb els que trastejo i als que fico i trec gens. Coneixeu la importància que té per mi l'amistat i és precisament així perquè quan he provat la vostra he vist que no podia viure sense ella, i encara menys treure'm un doctorat que tothom sap que és més complicat que (sobre)viure. Gràcies infinites, i em quedo curt.

Ara fa més de quatre anys escapava la mort per entrar a fer un doctorat. Ironies de la vida, sortir-te'n d'una per ficar-te en una altra! Ara ja puc dir que em disposo a escapar també del doctorat i és gràcies a totxs vosaltres. Al final d'aquesta etapa, tal com em passava poc abans d'entrar al quiròfan, puc dir, igual que Sacks, que el sentiment que predomina en mi és el de *gratitud*.

Max Carbonell Ballestero

Abstract

Synthetic Biology is a relatively new and multi-faceted interdisciplinary emergent field of research that combines biology with technology in novel and exciting ways. One of its main branches aims to see living systems engineered in a rational and straightforward bottom-up approach, like in other engineering disciplines. The inherent complex nature of living systems turns them into a difficult and challenging substrate where to apply common engineering principles such as *standardization*, *abstraction* and *modularity*. Efforts to overcome these limitations and adapt such principles for working upon living systems have been devoted, though yet with relative success. *The aim of this Thesis is to critically explore what is Synthetic Biology and how far it is from a veritable engineering discipline*. In this Thesis, we first present a review that thoroughly explores and discusses this scenario. Then, we present two works that shall contribute to this ambitious and hard goal. First, within the context of standardization, we address the need for better genetic parts characterization by providing an example of a biologically grounded framework inspired by classical enzymology theory. Second, and in relation with the principle of modularity, we provide a theoretical framework, in this case inspired by the Ohm's law of electric theory, that describes the unintended coupling of the coexisting genetic loads within a given host cell due to sharing a limited common pool of machinery and resources. Together, both works contribute, on one hand, to increase our understanding of the organizing principles of living systems, and on the other hand, to improve how engineering principles are applied to synthetic circuit design. Finally, these works emphasize the need to find better experimentally backed-up theoretical frameworks or models that should allow us to jump from the current time-consuming, trial-error and *ad hoc* Synthetic Biology to a well-established engineering discipline as fruitful and efficient with the living systems realm as other engineering disciplines are.

Resum

La Biologia Sintètica és un camp de recerca emergent relativament nou i multi-facètic que combina la biologia amb la tecnologia de formes innovadores i emocionants. Una de les seves principals branques té com a objectiu aconseguir ingenieritzar els sistemes vius des de sota de manera racional i senzilla, tal com passa en altres tipus d'enginyeria. La naturalesa inherentment complexa dels éssers vius els converteix en un substrat difícil sobre el qual aplicar principis d'enginyeria com l'*abstracció*, l'*estandarditzación* i la *modularitat*. S'han dedicat esforços per superar aquestes limitacions i adaptar aquests principis perquè funcionin sobre sistemes vius, tot i que encara que amb un èxit relatiu. *L'objectiu d'aquesta tesi és explorar críticament què és la Biologia Sintètica i quan lluny está de ser una veritable enginyeria*. En aquesta tesi, primer presentem un article de revisió que explora i discuteix a fons aquest escenari. Després presentem dos treballs que han de contribuir a aquest ambiciós i difícil objectiu. En primer lloc, en el context de l'estandarditzación, adrecem la necessitat d'una millor caracterització de les parts genètiques oferint un exemple de marc teòric amb fonaments biològics que esta inspirat en teoria enzimològica clàssica. En segon lloc, i relacionat amb el principi de modularitat, oferim un marc teòric, aquest cop inspirat en la llei de Ohm de la teoria elèctrica, que descriu l'aparellament no intencionat de les carregues genètiques coexistents dins d'una cèl·lula hoste qualssevol degut al fet de compartir un conjunt comú limitat de recursos i maquinària cellular. Ambdós treballs contribueixen, per un cantó, a incrementar el nostre coneixement sobre els principis d'organització dels éssers vius, i per l'altre, a millorar com s'apliquen els principis d'enginyeria pel disseny de circuits sintètics. Finalment, aquests treballs emfatitzen la necessitat de trobar millors marcs teòrics o models recolzats experimentalment que haurien de permetre'ns fer un salt des de l'actual Biologia Sintètica *ad hoc*, farregosa i basada en assaig-error, a un tipus d'enginyeria ben establerta que pugui ser tan profitosa i eficient en el reialme dels éssers vius com ho són les altres enginyeries.

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PREFACE

The last century has been a privileged witness of how scientific and technological progress has increased enormously. Throughout the history of science there have been thousands of notable discoveries, as it is nicely illustrated in Bryson's book *"A short history of everything"*. Energy -and the ways to control it- would be one of such milestones in the first half, the advancements achieved in computing and telecommunications during the last decades would be another.

However, I would like direct the attention here to something closer to the topic of this Thesis. Reported by the UNESCO as *"the most revolutionary developments in the second half of the last century"*, besides the rise of microcomputers and the Internet, the emergence of genetic engineering and biotechnology was also included as the other major achievement of the aforementioned technological and scientific progress [UNESCO, 2000]. It is worth stressing that we are living an acceleration process in Science. Science and Technology are dramatically changing our lives fast and deep. Now, at the beginning of the 21st century, we start to see the fusion of apparently distant disciplines such as biotechnology and computation, opening a new window of unimaginable impact to our lives. I hope that the first half of the 21st century we might become more than privileged witnesses of another revolutionary and game-changing development under the name of **Synthetic Biology**.

This field was was born with the dawn of the new millennium drawing inspiration from the knowledge and methodologies of different sources, including biotechnology and computational biology among the most relevant ones [Benner and Sismour, 2005, Cameron et al., 2014]. The Thesis I

present is immersed in this challenging field and provides a set of results that should help the development of this appealing discipline towards a veritable engineering methodology.

Synthetic Biology is law-breaking in Biology in the sense that it offers a novel approach for understanding life that goes beyond the traditional one in biology, based on observation, analysis and reverse engineering. Somehow inspired by Feynman's famous quote[1] "*What I cannot create I do not understand*", Synthetic Biology pursues to gain understanding about life by trying to build it from scratch, either by modifying already existing forms of life or by creating new ones [Elowitz and Lim, 2010, Csete and Doyle, 2002]. The introduction of synthesis practices in Chemistry during the 19th century revolutionized the discipline, leading to a deeper understanding of the fundamental principles governing Chemistry and to the emergence of modern chemical and pharmaceutical industries [Yeh and Lim, 2007]. Taking inspiration from the analogy with Chemistry, looking at the advances in Biology and Biotechnology, a question arises: *Could Synthetic Biology follow the path of Synthetic Chemistry and propel our understanding of living organisms and of the ways to engineer them*? Until now we have just begun to glimpse its potential impact, ranging from human health to the environment and even industry [Khalil and Collins, 2010], but *how far can we go*?

As an emerging field, it seems clear that Synthetic Biology holds more than one scientific approach. Among them, there is one that occupies a central position in this Thesis: the efforts to turn Synthetic Biology into a veritable engineering discipline. This was the beginning of the story I wanted to critically explore in this Thesis. Drew Endy and Tom Knight, both from the MIT, proposed the application of well-known engineering practices and principles to biology [Endy, 2005]. Framed in the history of the foundations of these disciplines, this Thesis is precisely aimed at exploring the application of fundamental engineering principles to living systems. In an ideal scenario, Synthetic Biology should allow for the ra-

[1]This quote was found written on his blackboard the day of his death. Obviously, it did not referred to Synthetic Biology, but it has been frequently used by synthetic biologists as a source of inspiration since the inception of the field.

tional and straight-forward design and construction of biological systems, as happens in other engineering disciplines with non-living matter.

However, it is worth stressing that this is an ambitious goal for a serious discipline, especially considering that, until recently, engineering has been mainly performed upon inanimate objects. There are various reasons to explain this: living systems are much more complex and unpredictable, and furthermore, our understanding of them is more limited compared to other physical systems. Hence, this new situation poses a set of new engineering challenges that need to be addressed. Several significant initiatives have been directed towards this goal with some success, working on the lines proposed by Endy, such as the problem of part characterization and the system's scalability, just to mention some of them [Endy, 2005, Heinemann and Panke, 2006, Andrianantoandro et al., 2006]. However, we are still far from a desirable state in which routine and efficient design and construction of biological systems is the rule and not the exception.

Actually, as a young synthetic biologist who started his career a few years ago, I have arrived at the same conclusion. This Thesis represents somehow my personal path within this field. After a large number of attempts to design and construct quite simple genetic devices, my rate of success was clearly not equivalent to other engineering disciplines. If this would have happened exclusively to me, I would have thought that it was because of my inexperience. However, I came to realize that I was not alone and, actually, I prefer to think that we are at the beginning of something big and that I am participating in this beginning.

There were, and still are, a lot of open questions and difficulties that synthetic biologists must face everyday, many related to the pillars that are supposed to sustain Synthetic Biology as an engineering discipline [Kwok, 2010]. Hence, I decided to slightly shift my research focus with the goal of bringing Synthetic Biology a little bit closer to other engineering disciplines by reinforcing its fundamental principles.

Therefore, in this Thesis I present a reflection of what is Synthetic Biology, where it comes from, where it is going and where I think it should go. Following this line, I present two contributions to that ambitious goal

that I think might be useful. Conscious of the importance and potential of this discipline, this work is humbly aimed at underpinning its foundations.

* * *

I would like to finish this preface with a general and personal reflection. The impact that science and technology has had in the world is profound. However, such impact has not always been positive, especially when they have been misused by societies who sought to ensure their superiority over others. If you look at it from a mid-term perspective, despite a number of negative outcomes that should not be forgotten, we can state that its net effect upon humanity has been beneficial. However, with regards to the planet's situation, and indirectly ours too, we are so aware of our (negative) impact on it that some authors dare to refer to the current geologic time as *The Anthropocene* [Crutzen and Stoermer, 2000].

In light of every new discovery and every potentially transforming technology, like Synthetic Biology may be, we should act with caution and engage in (bio)ethical discussions around the challenges and potential dangers that emerge from such discoveries, within the scientific community but also within society, making democratic decisions accordingly. I am aware of the existence of several initiatives, debates, meetings and publications that deal with this topic. Although this issue is not explicitly treated within the work you have in your hands, it is something that often occupies my thoughts and conversations, and that I do not forget in my daily scientific practices.

Max Carbonell Ballestero
La Habana, Cuba - June, 2015

Chapter 1

INTRODUCTION

When I speak of reason or rationalism, all I mean is the conviction that we can learn through criticism of our mistakes and errors, especially through criticism by others, and eventually also through self-criticism.

On freedom. All life is problem solving - Karl R. Popper

1.1 Synthetic Biology, a brief overview

Synthetic Biology is a true scientific discipline of the 21st century: it moves between blurred boundaries, evolves fast and sells itself well. Ever since its appearance, between the end of the last century and the beginning of this new one, has attracted a lot of attention, both from the scientific community and the public as a whole [Moore, 2006, Markoff, 2014]. The possibility of deliberately creating life from scratch has for a long time stimulated human's imagination, ranging from philosophers like Descartes and fiction writers like Mary Shelly to scientists like Stephane Leduc, Craig Venter or many others[1] [Lewontin, 2014, Ball, 2011, Rasmussen et al., 2009]. In any case, all the scientific and technological progress achieved during decades closes us in on the ability to conquer this goal, with potential consequences both good and bad [Krauss, 2010, MacDonald et al., 2011].

Synthetic Biology is not a well-defined discipline. In fact, its definition is a matter of a strong and continuously evolving debate, not only among the scientific community but also in human sciences, arts and politics [Wikipedia, 2016]. Paying only attention to the name itself, one can easily see that it is composed of two apparently contradictory elements: synthetic (i.e. man-made) and biology (i.e. natural, not man-made). For the general public this can be too broad and ambiguous. Indeed, these two words do not provide a clear clue of what this discipline is about, but they do create some kind of expectation. However, with regards to the scientific community, it is widely accepted that under this name there lie a number of slightly different initiatives that share some common ground.

But, *what makes Synthetic Biology "special"*? As I have suggested in the Preface, the common leitmotiv that drives these different branches of Synthetic Biology is "*build to understand*" [Elowitz and Lim, 2010] (see **Figure 1.1**). The reason why there are now scientists who think that

[1] Descartes conceptualized living machines, Shelly imagined Dr. Frankenstein, Leduc wrote the first scientific book on the topic, entitled "La Biologie synthetique", and Venter has pioneered both the uncovering of the Human Genome and the creation of the first living cell with a synthetic genome.

INTRODUCTION

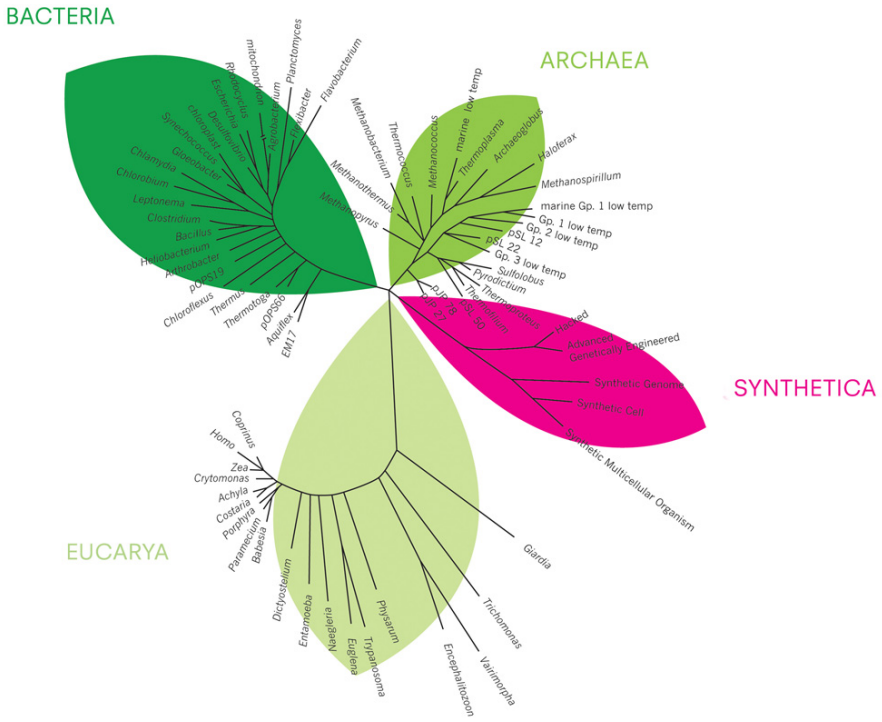

Figure 1.1: **The Synthetic Kingdom**. This hypothetical tree of life displays three main branches -*bacteria*, *archaea* and *eucarya*- and one extra branch -*synthetica*- that comprises all the organisms to which Synthetic Biology may have gave birth. *Adapted from [Baldwin et al., 2012].*

we are ready to attempt to build life from scratch is found on the great deal of scientific progress made so far. Watson and Crick's discovery of DNA structure and the revolution of genetic engineering of the 70s and 80s [Watson and Crick, 1953, Jackson et al., 1972] have been two cornerstones that have facilitated the enormous increase in the understanding of cells at a molecular level we have seen during the last decades. Another early essential contribution was the clever Jacob and Monod's paper published in 1961 [Jacob and Monod, 1961]. In that paper they provided a view of genetic regulations as a sort of switches: a very primordial perspective of what is now setting the so-called field of cellular computation. Later on, the onset of a discipline like Systems Biology and the growing potential of continuously new appearing computational and experimental tools contributed to reinforce the basis upon which Synthetic Biology could later emerge [Kitano, 2001, Westerhoff and Palsson, 2004].

And, *what about Biotechnology? Was it not supposed to have to do with Engineering?* This is a big claim -probably controversial- defended in this Thesis. In this context, one of the major defiances is to establish solid engineering principles for Synthetic Biology. Nevertheless, the consequence of merging two -until now- distant fields, **Biology** and **Engineering**, implied the emergence of new challenges. Neither Biology has ever been thought or applied following engineering criteria, nor engineering has been performed on living systems instead on non-living ones (see **Figure 1.2**).

It is true that technology and biology have traveled together for a while within a discipline called Biotechnology. This experience has taught us a lot and has been an inevitable prior step of this new endeavor we are immersed in. However, it is worth stressing that what the presented approach seeks to do is to reach beyond biotechnology and (classical) genetic engineering[2]. In Synthetic Biology, the concept of 'engineering' is assumed in its totality. Thus, it is not only about using technology to slightly interact

[2]The term genetic engineering -or genetic modification- refers to the classical approach of Biotechnology that uses different techniques to (slightly) modify the genome of a given organism. It does not refer to the new engineering-oriented approaches done within Synthetic Biology

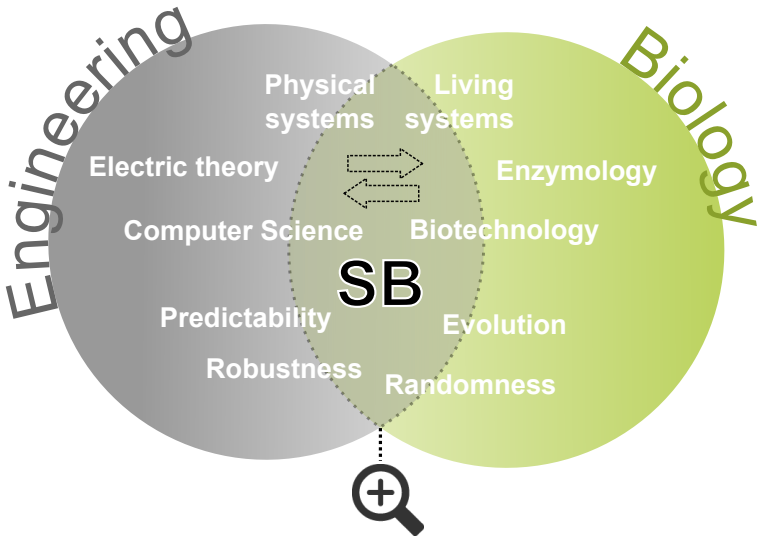

Figure 1.2: **Engineering meets Biology.** The branch of Synthetic Biology (SB) in which this Thesis is framed lies at the crossroads of biology and engineering. It implies the encounter between two very different approaches: one with a constructive nature, the other with an investigative one; one that mainly acts upon non-living systems, the other with living ones; one that seeks to create and built, while the other wants to understand. Approaches like Computer Science and Biotechnology started to cross the boundaries that separate both disciplines, yet not at a deep level. Synthetic Biology aims to go beyond these attempts and turn itself into an engineering discipline applied to living systems.

with the genome of an organism but, instead, it is about being capable of building-up complex genetic devices and systems based on a rational design, either from the vast catalogue of elements that nature has provided or from newly created ones.

The works published in the beginning of this century by Elowitz and Gardner can be considered as the initial efforts to conquer this goal. From then, molecular biologists, engineers and other scientists with different backgrounds got to work together and started exchanging knowledge and methodologies. The firsts results of this new interdisciplinary field did not take long to appear.

Thus, it was *Nature* journal that published in January of the year 2000 the two seminal papers that broke the ice of this nascent discipline. Both were genetic transcriptional circuits implemented in *E. coli*. On the one hand, Gardner and colleagues created a 2-gene bistable circuit composed of two negatively regulated promoters that were inhibiting each other [Gardner et al., 2000]. On the other hand, Elowitz and Leibler made a 3-gene network that displayed oscillations, also using three tanscriptional repressor systems [Elowitz and Leibler, 2000]. Both circuits were thought as proofs-of-principle and showed the capacity of this discipline to successfully design and build complex genetic circuits, merging model simulations with experiments. It is worth noting that both systems were designed applying the same design rules used in electronics. The optimal results obtained suggested that this could be the best way to design complex genetic devices.

After these two works, there have come many more proof-of-principles that have helped to widen the scope of this discipline. For instance, drawing inspiration again from electronics, several biological gates have been implemented that mimic the logic gates found in computing devices [Hasty et al., 2002, Moon et al., 2012]. The possibility of combining such gates and scaling-up the circuits allowing cells to act like small computers and perform complex tasks has met with several difficulties and is still an on-going endeavor, though there have also been some important successes [Regot et al., 2011, Macía et al., 2012, Ausländer et al., 2012, Purcell and Lu, 2014, Sardanyés et al., 2015].

With regards to the organisms susceptible of being engineered in the field, the implementation of synthetic circuits has not been limited to bacteria but it has expanded to other organisms. Yeast is another common workhorse of this discipline, as it has been for decades in the biotechnological industry. Most of the circuits that have been explored so far in bacteria have also been implemented in yeast cells, as well as other different architectures. Likewise, mammalian cells have been successfully targeted by synthetic biologists. Fussenegger and colleagues and other research groups have also implemented, for instance, logical gates (also known as "BioLogic Gates"), bistable circuits or oscillators, among others [Kramer et al., 2004a, Kramer et al., 2004b, Tigges et al., 2009].

With regards to the type of circuits being implemented, it is worth noting here that transcriptional circuits have not been the only tools that synthetic biologists have used. DNA is still the primary substrate upon which to engineer the circuits, but other promising paths have also been explored. RNA, for example, has shown itself as quite a versatile molecule that is able to interact with DNA, proteins and other molecules, which turns it into a tool with a lot of potential. For instance, riboregulators and ligand-dependent riboregulators have already been developed, allowing for a tight control of gene expression within synthetic circuits [Isaacs et al., 2004, Bayer and Smolke, 2005]. Moreover, other possible roles for RNA in Synthetic Biology are currently under exploration [Chappell et al., 2015].

Another tool that might be used for the engineering of synthetic circuits are proteins. They are even more versatile than RNA, as they can act at different temporal and spatial scales and exhibit an extraordinary molecular diversity that enables them to perform a myriad of functions. However, our still limited knowledge about their function-structure mapping makes them a difficult substrate for engineering. Although there have been a few successful examples, like rewiring signaling pathways or modifying the metabolic flux through a synthetic pathway, we are still at an embryonic stage [Grunberg and Serrano, 2010].

But cells offer additional layers that are neither the transcriptional nor the translational ones. As seen until now, one cell can host a new function-

ality. However, it is also possible to think from the multiple-cell perspective, to make cells to work together. Interestingly, T. Knight and R. Weiss pioneered the used of the quorum sensing (*QS*) machinery imported from *Vibrio fischeri* to coordinate groups of cells by using a set of chemical diffusible molecules known as lactones [Weiss and Knight, 2000]. Thanks to this system it has been possible to coordinate oscillations or to produce spatial patterns among groups of cells [Danino et al., 2010, Basu et al., 2005].

Finally, from the (bio)engineering perspective, computer and mathematical models have also made part of the advance of synthetic biology to offer a sort of science with predictable nature. As examples of the efforts of modeling in this field, just to name only a couple, we find works like the advances in cell computation [Regot et al., 2011] or in applied research towards the cure of diabetes [Miller et al., 2012].

1.2 Engineering principles for living systems

After some examples, one can envision the potential of Synthetic Biology, even more when considering its short lifetime and that it deals with an appealing topic (i.e. creation of life). In this regard, it is easy to understand the hype associated with this discipline and the media coverage it has attracted [Moore, 2006, Markoff, 2014, Lewontin, 2014]. Nevertheless, not all are good news in Synthetic Biology. A discipline that aspires to be as fruitful and efficient as other engineering disciplines cannot afford to rely on *ad hoc*, time-consuming and trial-and-error processes of design and construction. And, in fact, if we review most of the examples explored until now, we would see that these have been accomplished thanks to such kind of processes that differ from other engineering disciplines [Arkin and Fletcher, 2006, O'Malley, 2009].

It is worth stressing that this way of working is more like handcrafting with cells than engineering them. A lot of time, money and human resources are required for designing devices that, in the end, have limited application. This is in part due to its *ad hoc* specific nature of design, based on our still limited capacity of tuning circuits and combining them with other devices. Besides, serious constrains for the scalability of gene circuitry exist [Lucks et al., 2008].

Thus, in parallel to all the projects and efforts devoted to the creation of ever more complex and appealing genetic devices, there is a genuine effort to establishing Synthetic Biology as much as possible as a truly engineering discipline, where each new device should not be treated as a new problem.

D. Endy was a pioneer in stressing the need for an engineering framework in Synthetic Biology. In 2005 he published a paper that laid the ground for Synthetic Biology to get close to becoming an engineering-oriented discipline. Entitled *Foundations for engineering biology*, in this work he proposed the application and adaption of 3 fundamental engineering principles to biological systems, namely *standardizations*, *abstraction* and *modularity* (see **Figure 1.3**) [Endy, 2005]. This idea was soon after reinforced by other authors [Heinemann and Panke, 2006, Andrianan-

toandro et al., 2006], thus constituting a major avenue of the development of Synthetic Biology.

The first principle, *standardization*, is the product of an agreement between the different players within the community. Such community decides a set of rules, processes and methods. These are considered in order to allow a proper and collaborative dissemination and production of knowledge related to the discipline and how it has to be developed. Hence, standardization is one of the key elements that permits an engineering discipline to rapidly grow and produce, it helps all the participants to somehow speak the same engineering 'language', collaborate and reuse what has been previously built by others [Endy, 2005, Arkin, 2008].

Therefore, as in other engineering disciplines, technical standards[3] are a must. Its use should span different scales: the definition of a DNA fragment or part, a suitable annotation in repositories and the documentation of a protocol of use, all take part in the standardization processes. For instance, with regards to the 'parts', its characterization is an important issue: how to define the different synthetic 'parts' or building blocks, how to characterize them, under what specific operating conditions, and so on and so forth [Canton et al., 2008]. Moreover, it seems quite obvious the need for repositories or other platforms that allow the sharing of such synthetic parts and all the necessary information related to them, as well as the ways to work with them. Following this line, the '*Registry of Standard Biological Parts*' (*RSBP)*, hosted at the MIT and led by Endy himself and other prominent synthetic biologists, has constituted a key initiative for the development of this field [iGEM, 2004].

With regards to the second principle, *abstraction*, it should help synthetic biologists to deal with the high degree of complexity of the systems they work with. It is the process of selecting all the key features needed to work with the system, leaving aside unnecessary details or redundant information. The result is a simplification of reality -a model- more affordable for any given scientist. Furthermore, it is worth noting that abstraction is tied to the concept of hierarchies. Given the size and complexity of

[3]Technical standards are guidelines that include all the necessary rules, specifications and details that the engineers must follow in the engineering process.

synthetic biology devices and systems, it is useful to organize its information in different levels or layers. Once this is done, the different levels can be dealt with independently and only little or no exchange of information between these levels is needed, which facilitates the management of each level. Hence, in Synthetic Biology the abstraction hierarchy often drawn is the following: from DNA to parts, to devices, to systems, to hosts (i.e. synthetic cells). [Endy, 2005].

Finally, the last principle, *decoupling* or *modularity*, refers to another fundamental feature of engineering disciplines. It is about the possibility of separating a complicated problem into simpler sub-problems. By doing so, these can be easily worked on with independence and thereafter re-combined to produce the predicted functioning whole. For instance, modular systems can be found in the engineering realm in all the designs in electronics [Endy, 2005].

Regarding to biology, although the existence of modularity has been proposed [Hartwell et al., 1999, Purnick and Weiss, 2009], as it occurs in protein-protein interaction networks [Rodriguez-Caso et al., 2005] and metabolism [Ravasz et al., 2002], it does not seem good enough for allowing an easy engineering of living systems. A property that is intimately linked with modularity is that of *orthogonality*[4]. This concept refers to the independent nature of the different modules, to the fact that they do not affect or interact with each other unless designed and built to do so.

These properties are essential for any successful building-up process that one would expect from an engineering field. However, as we shall see, designs often fail due to the lack of modularity and orthogonality [Kwok, 2010]. Among the factors reponsible of these failures we find *cross-talk*[5], metabolic load[6], noise, variability, nonlinearities or emergent phenomena.

[4]Orthogonality: it implies a factual independence between otherwise co-existing systems or elements, it refers to modules that do not interact or interfere with each other.

[5]Cross-talk: the process by which one signal interacts with one unintended or undesired target (e.g. in *QS*, when one lactone activates another lactone's receptor).

[6]Metabolic load: synthetic circuits expression, and in general terms the (over)expression of a foreign gene, has negative effects on host cells (e.g. growth rate decrease) due to consumption of host resources.

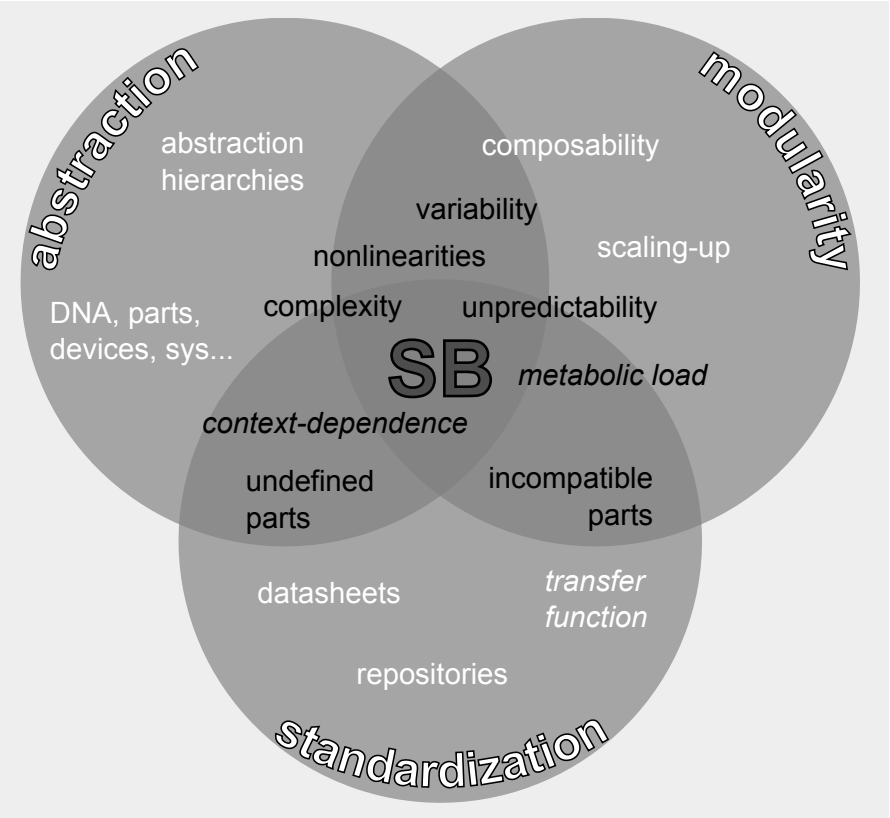

Figure 1.3: **Principles and related problems in Synthetic Biology.** This figure introduces the three main engineering principles that D. Endy proposed to be adapted and applied to living systems. Furthermore, it shows some of the issues that are related to them (in white) and some of the main problems and challenges that prevent the success of this approach (in black). In *italics*, either in white or black, some of the issues addressed by the works included in this Thesis.

1.3 Problems and Challenges

Over more than a decade after the launch of the field several attempts have been done to bring Synthetic Biology closer to the engineering realm. The goal has revealed more difficult than what the initial hype that surrounded the emergence of this field once suggested. Especially regarding the establishment of engineering principles that are supposed to enable the routine and straight-forward rational design and construction of synthetic biology devices [Serrano, 2007, Gardner, 2013]. As Timothy Gardener has recently pointed out *"the field has lost sight of the fact that its founding premise has not yet been validated"* [Gardner and Hawkins, 2013]. The sad truth is that today, as ten years ago, we are still in a similar situation: much work is still needed to arrive to the desired point in which Synthetic Biology behaves as an engineering discipline and several are the forces or factors that oppose resistance to such process.

The inherent complex nature of living systems is, in fact, the major barrier. Unlike normal engineering substrates, i.e. inanimate objects that are subject to well-known laws of physics and that display fairly predictable behaviors, living systems represent a harder challenge for engineering. Living organisms present much more complexity, physical variation, nonlinear behavior, noise, and in addition to physical laws they are also subject to evolution. Predictability is essential for any engineering discipline but when it comes to living systems our capacity in this aspect is rather limited.

Hence, the first thing that needs to be addressed is our lack of understanding of these systems, especially at the molecular level. Progress regarding this aspect has been enormous during the last decades, both in terms of knowledge and the techniques and methods needed to deal with them. In fact, our tools to manipulate these systems have often outpaced our knowledge of them. For instance, we can easily sequence, synthesize and, since recently with CRISPR technology [Jiang et al., 2013] even alter DNA at will, but we are still far from knowing all the secrets it hides. We know how to transform it but, overall, this lack of knowledge could be considered the main cause responsible for not going as fast as one of

the founders of Synthetic Biology initially claimed [Endy, 2005, Kwok, 2010, Gardner, 2013].

This lack of understanding is somehow related to other problems of the field that should also be addressed. The main ones were wisely highlighted by R. Kwok's famous paper *Five hard truths for Synthetic Biology*, namely: the undefinition of many parts (i), the unpredictability of the circuitry (ii), the unwieldy complexity (iii), the incompatibility of parts (iv), and variability of the systems (v) [Kwok, 2010].

All this leads us to a hard truth, which is that Synthetic Biology still requires extensive hand-craft tuning and lacks of predictable design capabilities. In other engineering disciplines there exist platforms that provide the set of standardized elements and tools that are needed for building up the devices. In Synthetic Biology, as mentioned before, there are similar initiatives but they are still far from satisfying its goal. When trying to build-up complex devices from the set of parts these provide, the most common outcome is highly unpredictable and, again, several cycles of re-design, re-tuning and re-building are required. This may be the reason why many synthetic biologists end up choosing the outdated trial-and-error style.

The responsibility for this situation is usually attributed to a bad characterization of the parts, however, many times there is no objection to the quality of the data. Then, *are we wrong somewhere else?* If the characterization is not properly achieved, our predictability is surely going to be compromised. *How should a device's characterization be properly performed? How may it be improved?*

Hypothetically, if all devices and modules were perfectly characterized, this should allow synthetic biologists to exploit the full potential of Synthetic Biology by combining such modules and devices to create more and more complex systems. However, it has not happened, at least not at the rhythm it was expected. There are several factors that might, and actually do, compromise the predictability of synthetic biology designs [Kwok, 2010]. Among these factors there are those that have to do with the context in which the circuits perform their function, either in terms of the host in which these are embedded or regarding to environment in

which they have to work [Cardinale and Arkin, 2012].

This context, which is often poorly known, understood and controlled, lies usually behind most synthetic biology device failures. Noteworthy, such failures appear more frequently when large circuits are assayed or when scaling-up designs. In these cases, besides blaming the context in a general way as responsible for such failures, another concept is used, linking these failures to the limited capacity of the host cells and using concepts like *metabolic load* or *metabolic burden*. These concepts describe the fact that cells see their usual performance altered due to the presence of the circuits that may force them to enter into a certain fatigue state [Glick, 1995, Chen and Silver, 2012]. Although these situations are common in the field, they are not yet well enough understood, not to say predicted or quantified.

Bearing all this in mind, along this Thesis I shall stress that the lack of theoretical frameworks to guide both the process of characterization of synthetic devices and the interaction of these foreign devices with its host are two essential issues that compromise predictability and modularity in the engineering processes.

Content Outline

At this point, I shall formulate the different objectives this Thesis pursues and that precede the works that are included in this PhD Thesis. After the Objectives chapter and before entering into the results of our research, I include a chapter of **Methodological Considerations** in which I shall explain which is the methodological approach used to pursue the above-mentioned objectives and why. Thereafter, in the **Results** chapter, I provide the main results we have obtained during these years in relation to the objectives we set.

The Results chapter is divided in three sections, one for each objective. In each section I present a summary of the results and a manuscript that backs them up. The first manuscript is a review that has not yet been published. The other two manuscripts are original articles that have been recently published in the journal *Nucleic Acids Research* [Carbonell-Ballestero et al., 2014, Carbonell-Ballestero et al., 2016]. Next, there is a **Discussion** chapter that is divided in four sections, one for each three results sections, in which I discuss the main results from a general perspective, and one with final general considerations or thoughts. After this discussion, the reader will find a **Conclusions** chapter in which the main conclusions corresponding to each objective will be stated. And last, an **Annex** chapter in which I briefly introduce another work I have participated in.

Chapter 2

OBJECTIVES

OBJECTIVES

Given all what I have just exposed in the previous chapter, in this PhD Thesis I have aimed to shed light on some of the open issues which have already been pointed out. In particular, I have first addressed the need of properly framing the topic, the engineering-oriented branch of Synthetic Biology, providing a firm background to support the Thesis. This should help the reader to understand its motivation and to adequately evaluate the context and significance of the further results included. Thereafter, I have directed the attention towards the scientific core of this Thesis, which is to try to reinforce and expand the theoretical and experimental corpus that should sustain Synthetic Biology as an engineering discipline.

Hence, the **main objective** of this Thesis has been to reinforce and improve the engineering foundations of Synthetic Biology, ranging from a conceptual perspective to practical implementations.

Following this line, the main **objectives** addressed in this PhD Thesis are:

O.1. Explore the field of Synthetic Biology and how it unfolds as an engineering discipline.

O.2. Explore how to improve standardization via better characterization of the synthetic devices.

O.3. Explore circuit-host interactions and its relation with the lack of proper modularity and the metabolic load.

Chapter 3

METHODOLOGICAL CONSIDERATIONS

Intuition and concepts constitute, therefore, the elements of all our knowledge, so that neither concepts without an intuition in some way corresponding to them, nor intuition without concepts, can yield knowledge... Thoughts without content are empty, intuitions without concepts are blind... Only through their union can knowledge arise.

Critique of pure reason - Immanuel Kant

In this chapter, I provide an overview about the general methodological approach that has been followed during this Thesis. It consists in the combination of theoretical and experimental approaches and is typical of disciplines like Systems or Synthetic Biology [Kitano, 2002]. Considerations about such combined approach are focused on the reason *why* we have chosen it. Furthermore, some general insights about *how* it has been developed, both at the computational and experimental level, are included. However, full instructions and specifications are not detailed, as they are already present in the Materials and Methods section of each article.

The idea of combining mathematical models with experiments is a desirable trait in any scientific discipline, though it has not always been possible. This is actually reflected in the evolution of Molecular Biology and Biotechnology to Systems and Synthetic Biology. These last disciplines cannot be understood nowadays from a traditional perspective that only contemplates either work in a theoretical branch or in an experimental one, but from a new perspective that results from the combination of both. Indeed, the quote that opens this chapter was wisely chosen by Serrano and colleagues to introduce their review about such combined approach entitled *From in vivo to in silico biology and back* [Di Ventura et al., 2006]. As they illustrate in the paper, this sentence makes sense if we consider '*intuition*' as empirical evidences (i.e. facts) and '*concepts*' as the laws or principles that are derived from them or cause them. Both are needed and both complement each other.

As any researcher knows, the scientific method is an ongoing endeavor that gets us closer to the acquisition of knowledge. The method is conceptualized by a cycle: we usually start by setting an hypothesis, then we decide the appropriate methodology to make experiments and measurements to test it, and, after, we analyze the results and further validate or discard the hypothesis based on them. Finally, depending on whether the hypothesis being validated or not, we close the cycle by re-formulating the hypothesis -if it is wrong- or by generating new hypotheses to address new questions, which often emerge from the things we have just found (see **Figure 3.1**).

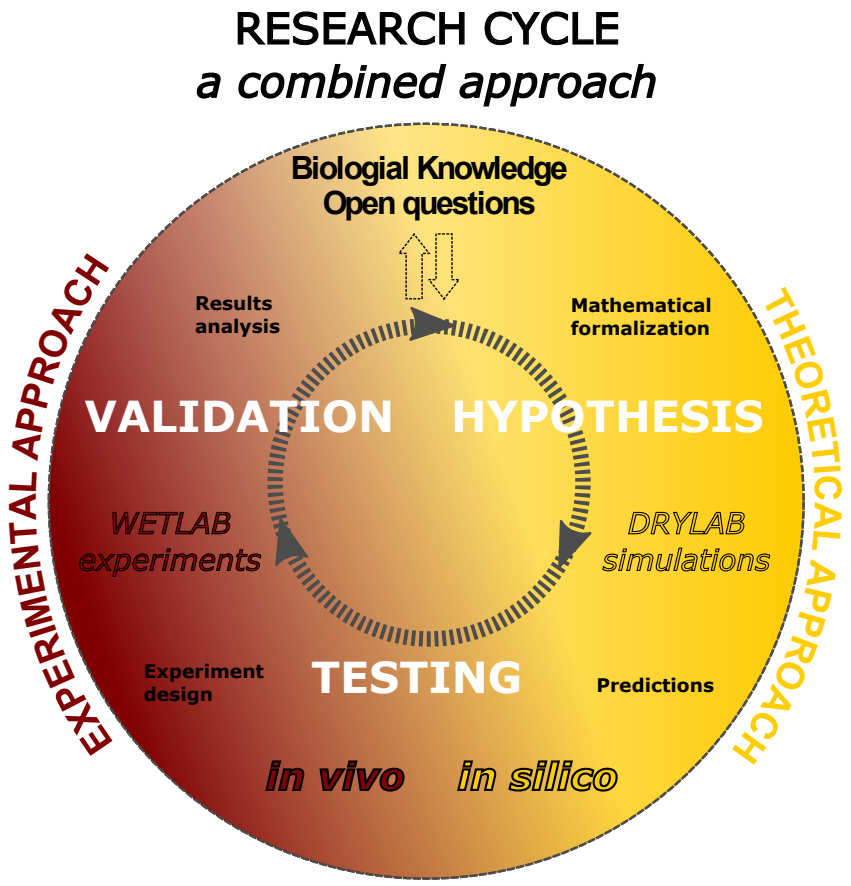

Figure 3.1: **Research Cycle.** ***A combined approach.*** This figure illustrates the classic research cycle (hypothesis, testing and validation) overlapped to the combined approach used in this Thesis, typical of Systems and Synthetic Biology: *in silico* and *in vivo* or *drylab* and *wetlab* (red and yellow). *Modified from [Kitano, 2002].*

Such process might take place only in the theoretical arena, in which we can (virtually) test the hypotheses *in silico*, or in a separate way at the experimental one, where we test them *in vitro* or *in vivo*. Yet, another option is available: directly combining both approaches. This combined approach might have synergistic effects in both process. This approximation helps to complement experimental results with theories of the underlying mechanisms at play. In this sense, mathematical models combined with computational tools might be of great help in order to understand the huge complexity of the biological mechanisms [Di Ventura et al., 2006]. This is not only due to technical issues, as computer tools might accelerate a lot of processes, but also because with these tools scientists are able to conceive, model, simulate and test a myriad of (alternative) scenarios, some of them that are even hard to imagine or conceive only with their minds. On the other hand, as models make sense if they are able to describe and predict reality, enriching or feeding our '*concepts*' with an empirical feedback (i.e. *intuitions*) is a good way to improve them.

Hence, our approximation consists of merging theory with experiments, i.e. *wetlab* with *drylab*, in a back and forth synergistic process that overlaps with the classical research cycle (i.e hypothesis, testing, validation), as shown in **Figure 3.1**. The idea is to make more meaningful and biologically informed models that might help us to better understand, describe and predict genetic circuits and the whole cell's behavior. At the same time, by experimentally testing our models we should be able to extract relevant information that should help us to enrich them and improve its predictive power and reliability. Altogether should allow us to gain more insight about the underlying ruling mechanisms and to confirm its validity [Kitano, 2002].

In the next two sections, both arms of our approach are going to be exposed. We will start first with the theoretical one in which the reader will find a general explanation of the mathematical modeling and a discussion of which kind of modeling methods we have used and why. Thereafter, in the second section, I will focus on our experimental approach. I start by providing a brief overview of the existing methods for building syn-

thetic circuits, especially of the one we have used. Later on, other related issues such as the model organism we have employed, the molecular biology techniques we have performed or the measurement methods we have used are also included.

3.1 Theoretical Approach

One of the cornerstones of science is the use of mathematics in order to model and understand the world. Ever since its appearance, mathematical models have become an essential tool for a lot of different scientific and engineering disciplines. In life sciences, for instance, they are an irreplaceable weapon for disciplines like Syntehtic Biology and Systems Biology (see **Figure 3.1**) [Zheng and Sriram, 2010, Sobie et al., 2011].

With models we try to capture the relevant principles and essential features that we think might govern and determine the behavior of the systems under study, while getting ride of all the unnecessary information. Ideally, besides of describing reality -or the hypotheses we might have regarding to it-, models should also have a predictive character.

Now, it is worth to examine in more detail what are models mainly composed of. On the one hand, mathematical models are grounded on a set of *assumptions*: simplifications of reality that define the boundaries of what we consider that has to do with our model. These are conditions we impose and assume that are true. For example: the number of molecules is large enough to neglect stochastic fluctuations or the concentration of the substrate is much bigger than the concentration of the enzymes. It is worth stressing that, despite by definition these assumptions are true in our model, they are not necessarily so in the reality.

On the other hand, models have -or are driven by- a set of *rules*: the mathematical description of the dynamics of the elements composing the system and that determine its behavior. Everything must be defined, even when the behavior of the elements is random. In any case, models should be based on the existing knowledge. Sometimes, we might just want to verify if a simple rule, excluding all the details, is enough to (re)produce one particular behavior. Another times, we might want to mimic reality by introducing all the available information of a given system. In fact, none of these two visions is wrong -or true at all- but they have different purposes, weakness and strengths.

As we have seen, together, *assumptions* and *rules* conform the skeleton of our models. Such models are somehow, in turn, the workbench of

our reasoning. They are a key step for the rational understanding and design of complex systems and help us to formally connect the conception of a system with its physical realization [Kaznessis, 2007, MacDonald et al., 2011, Le Novère, 2015].

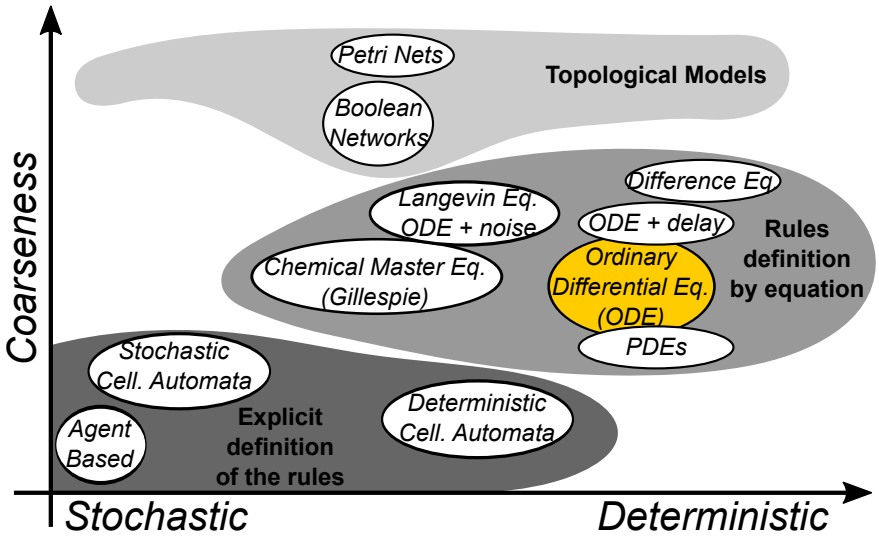

Figure 3.2: **Map of approximations in Synthetic Biology models.** This map shows different modeling approximations used in the field. Its position depends on whether they are stochastic or deterministic -or something in between- and on its level of coarseness. As explained in the main text, we have chosen a deterministic approach implementing *Ordinary Differential Equations* (*ODEs*) to describe with an intermediate level of coarseness (i.e. mesoscopic level) the rules that govern our system (in yellow).

So, when starting to asses from a theoretical perspective the hypothesis regarding to our system under study, we have to choose what kind of model suits it better. This decision might depend on factors such as the degree of detail we want to consider, the kind of processes we are dealing

with (e.g. dynamic or static) or the questions we might want to answer. Considering these issues, there are different types of modeling approaches from which to chose. In **Figure 3.2** the reader can see a map comprising several modeling approximations in Synthetic Biology, placed according to their level of abstraction (i.e. coarseness) and the type of mathetmatical and computational approaches used (i.e. deterministic or stochastic).

I shall now expose the approximation followed in the works presented in this Thesis. Regarding to the level of abstraction, in our case we built mesoscopic models. These allow us to properly describe the key biological processes related to the genetic circuits and its dynamics (e.g. transcription and translation) and incorporate and cover the main molecular species we want to study (e.g. transcript factors, signaling molecules, promoters, and so on). This level of concretion results from a compromise between a high level of precision and microscopic detail -which would render the models conceptually and computationally intractable- and a macroscopic detail that would be unable to capture any of the molecular processes of our circuits. A core idea must not be forgotten here: to understand reality do not build a model as complex as reality is.

Once the level of abstraction is already decided, we have to choose the type of modeling approach we should use. These approaches are mainly classified in two groups: *deterministic* or *stochastic* (see **Figures 3.2 and 3.3**) [Zheng and Sriram, 2010, Le Novère, 2015]. On the one hand, *deterministic* models describe the averaged behavior of populations of species that are composed by a high number of elements (e.g. molecules or cells). These systems are represented with analytical equations in which the variables usually represent the different interacting species and the parameters may account for different things, ranging from the kinetics of the reactions to the volume in which these take place. On the other hand, *stochastic* models describe all the elements of the species considered and the reactions are described in terms of probabilities instead of by deterministic equations. These kind of models are aimed at incorporating the inherent randomness or fluctuations of living systems.

Hence, while in deterministic models given the same initial conditions the results are always the same, stochastic ones cover the different pos-

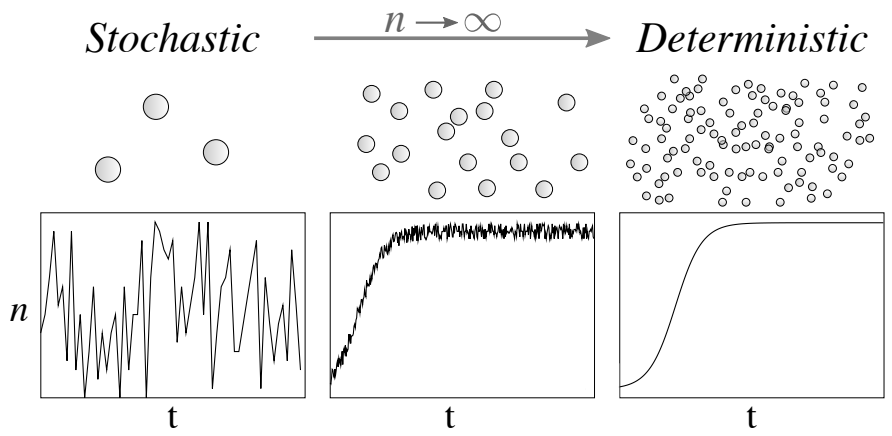

Figure 3.3: **Simulation types.** There are two main classes of simulations, either *stochastic* or *deterministic*. The former are used to model systems with low number of interacting particles in which random effects matter. As the number of particles increases, both approaches converge to the same result [Le Novère, 2015].

sible outcomes that might be produced due to random fluctuations of the interacting species. Nevertheless, the problem with stochastic models is that they are computationally much more demanding and, therefore, are only recommended with systems with low number of elements in which random effects might be expected. Stochastic models, however, tend to converge with deterministic ones as the number of elements increases (see **Figure 3.3**). In this Thesis, we decided to use deterministic models as the systems we are dealing with are composed by species with large number of components (e.g. molecular components at a cellular level).

Deterministic models are usually implemented through *Ordinary Differential Equations* (*ODEs*) to describe the time evolution of its composing species. However, rather than focusing on the dynamics of our systems we are interested in the equilibrium states they reach. This fact allows us to transform the set of *ODEs* into static or regular equations after imposing the equilibrium condition. By doing so, our models become much more easy to deal with and allows us to treat them analytically. In

our opinion the analytical treatment offers us the opportunity to get access to a more rigorous corpus of a discipline. In this case, *ODE* approximation provides a first step in this direction.

Finally, I would like to remind that the two research articles describing our theoretic-experimental approaches presented here have been precisely inspired by theoretical corpus coming from other disciplines. On the one hand, in the paper that deals with genetic devices characterization, the classical enzymological theory of Michaelis and Menten has been used as a guiding framework [Cornish-Bowden, 2004]. On the other hand, in the paper that deals with the genetic load, we shall see that our model converged to the well-known Ohm's law from electric theory, which has served us as a powerful analogy [Nilsson and A, 2011].

3.2 Experimental Approach

Coming back to our research process cycle, the other steps correspond to the experimental branch (see **Figure 3.1**). According to what has been devised and designed *in silico*, we must then construct or create its real or *in vivo* counterpart. Noteworthy, this is not a trivial step, as all the flexibility and speed existent within the computational arena disappears when it comes to the *wetlab*.

It is worth stressing that, as it happens with the theoretical models, the experimental approaches also require of a set of *premises*. These are boundary conditions imposed by the need of simplifying the experimental setup and due to technical limitations. Examples of such cases are found when we fix the temperature in a given experiment, use a particular bacterial strain or fix a given time for gathering measures. Indeed, experiments also constitute kind of simplifications of reality, though much closer to it than models. It is important not to forget this fact when contrasting experimental and modeling data and inferring conclusions from them.

Regarding to the first step, the construction of the circuits we have designed and modeled, there are different options available: *ad hoc* cloning with classical molecular biology methods (i), DNA synthesis in Biotech companies (ii) and standardized assembly methods (iii). The first option (i) is still used among synthetic biologists and other people working in the *wetlab*. However, as other options appear, its use diminishes due to their *ad hoc* and handcrafted nature, which is tedious, slow, expensive and hardly reusable [Sambrook and Russell, 2001]. The second option (ii) is still too expensive, especially when large constructs are to be created. Nevertheless, due to the fall of costs that DNA synthesis has experienced during the last years it has potential to become, sooner than later, an affordable option worth to consider [Ma et al., 2012, Kosuri and Church, 2014].

For these reasons, in the process of cloning we decided to use standard assembly methods (iii), which have become the choice of preference among many synthetic biologists. At this point, there are different alternatives, like one-step widely used assembly methods such as the

Golden Gate [Engler et al., 2008] or the Gibson Cloning [Gibson et al., 2009]. However, our choice was to use the BioBrick standard and cloning method for the step-wise assembly of parts [Knight, 2003, Ellis et al., 2011]. This method is complemented thanks to the 'Registry of Standard Biological Parts' (*RSBP*) that we used as the repository from which to mainly obtain the parts we needed (see **Figure 3.4**) [iGEM, 2004]. Besides, the community and philosophy that is tied to this option, so wisely reflected in the iGEM university competition, is indeed another reason to support this choice. Such community is a rich source that provides knowledge and know-how, whereas at the same time constitutes a nice endeavor worth to contribute to and be part of [Goodman, 2008, Smolke, 2009, Vilanova and Porcar, 2014].

As a result, virtually all the genetic circuits employed in the works presented in this Thesis were built with parts obtained from the *RSBP*. When the genetic parts we were interested in did not exist yet in the *RSBP*, these were designed and synthetized following the BioBrick cloning standard. As a consequence, they could be introduced and catalogued in the *RSBP* and further used in combination with the other parts and constructs present in the repository.

The *RSBP* is organized according to the abstraction hierarchy proposed by Endy and colleagues [Endy, 2005]. The 'parts' we mainly used, following their nomenclature, were promoters (constitutive or regulated, with different intensities), Ribosome Binding Sites (*RBSs*) (library of different strengths), coding sequences (e.g. reporter genes or transcript factors) and terminators. All these building blocks should be widely studied and characterized before its inclusion in the registry, besides being adapted to meet the registry cloning standards. Paradigmatic examples of such parts are the reporters, which in our case are genes coding for fluorescent proteins that come from other species. We used, for instance, the green fluorescent protein gene (*gfp*) that comes from the jellyfish *Aequorea victoria* [Cody et al., 1993] and that is broadly used in cell biology as readout of gene expression [Chalfie et al., 1994]. Another relevant examples would be the use of building blocks that belong to the *QS* machinery, such as the Lux promoter or the gene coding for the Lux Receptor,

which are part of a system widely used in Synthetic Biology devices, the lux-inducible-system [Miller and Bassler, 2001, Garg et al., 2014, Davis et al., 2015].

Noteworthy, besides the molecular biology tools are inherent to the Biobrick Assembly Kid, we have also used other classic molecular biology techniques when needed, such as digestion, ligation, electrophoresis gels, PCR and so on and so forth [Sambrook and Russell, 2001]. Among other things, these tools have allowed us to synthetize new parts and to confirm the success of the cloning processes. Furthermore, all the constructs that haven been used in the different experiments present in our papers have been validated (i.e. sequence confirmed) by Sanger sequencing to discard that any possible cloning error or mutation was present [Sanger et al., 1977, McGinn and Gut, 2013].

It is also worth to include here a comment about the methodology chosen to measure our circuits performance. In the approach of this Thesis we decided to get fluorescence measures using a micro-plate reader. This method allowed us to obtain an exhaustive tracking of gene expression and cell growth over time and at a population level with minor manipulation of cells cultures. Other alternatives such as Fluorescence-activated cell sorting (*FACS*) or microscopy, were discarded. Although *FACS* is another method that allows to get information from the expression of a cell population -and even at an individual-cell level- it requires more experiment manipulation and it hinders monitoring over time. Regarding to microscopy, it is highly recommended for single cell behavior but it is not suitable when we want to know the behavior of large population of cells.

Finally, it is important to highlight the model organism that was used and the reason why. The natural choice was to work with *E. coli*, the preferred and most widely used bacterial model organism since the onset of the molecular biology revolution around 1950s. Its robustness, versatility and ease of handling in the *wetlab* are characteristics that contribute to this choice. Furthermore, its biology, especially in terms of its genetic background and molecular machinery, is among the most studied and well-known ones [Blount, 2015]. All these issues makes it as the most preferred workhorse for hosting the synthetic circuits, as it has been

demonstrated since the birth of Synthetic Biology.

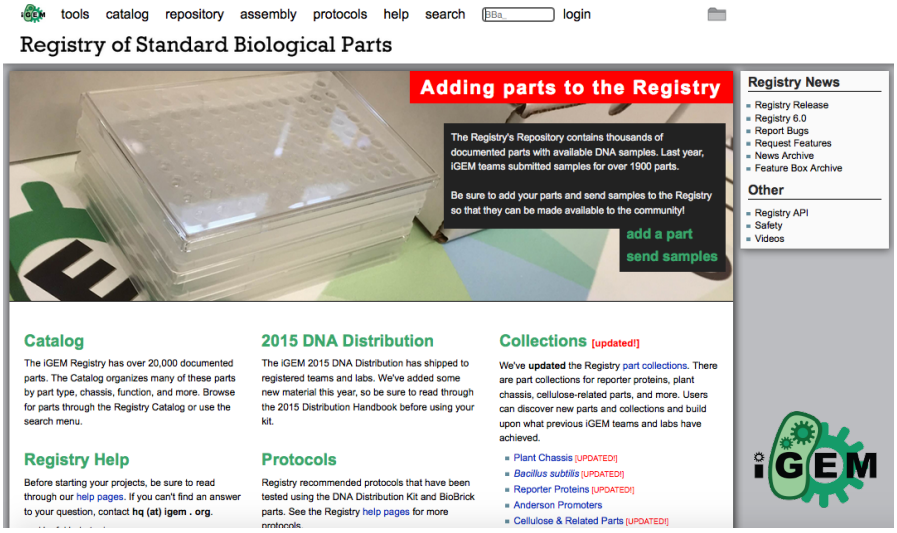

Figure 3.4: **Registry of Standard Biological Parts (*RSBP*)** This is a screenshot of the website of the *RSBP*, the repository from which most of the parts used in this Thesis to the assembly of the devices an systems have been obtained. The *RSBP* was founded in 2003 in the MIT and contains thousands of parts. The community built around this initiative is reflected in the iGEM Foundation's annual synthetic biology competition. The registry uses the BioBrick standard and provides many types of biological parts. Furthermore, it contains information about parts performance, know-how of different issues related with the field and a rich community to interact with. The website link is: `http://parts.igem.org/Main_Page` [iGEM, 2004].

Chapter 4

RESULTS

Result's Chapter organization and purpose

In this chapter I shall present the main results of my PhD Thesis. It is written in the format that attaches the articles as they have been published or submitted. Hence, and considering that I include three articles within this Thesis, the chapter is divided in three sections, each corresponding to one of the articles. The order in which they are presented corresponds to the logical order that guides this Thesis and is coherent with the objectives formulated: from a general conceptual overview to the two particular scientific findings.

In the first section the reader will find a *Review* of Synthetic Biology, which has not been published at the time of depositing this Thesis, that provides the background and context that help to situate the field and frame the other two works that are presented. These two *Original Articles*, published in *Nucleic Acids Research* journal, conform the following two sections. Hence, in the second section I include a research article published in the end of 2014 that focuses on the characterization of a very relevant and widespread inducible system. Finally, in the third section, I present another research article published approximately one year later that is about the role of genetic load in Synthetic Biology device's behavior.

Overall, this Thesis describes a number of contributions to the advance of Synthetic Biology towards the goal of turning it into a true engineering discipline or getting it as close as possible. Besides, it also provides a reflection about the nature of this discipline, from its roots to its actual fruits, *from concept to practice*, and discusses which could be, in its author's opinion, good steps towards this goal, It reflects on which is its contribution to the advance of scientific knowledge in this field. Hence, what is presented here is a consensus between the need of accurately reflecting the scientific findings that it contains and that of framing these findings within the scientific knowledge map.

4.1 On Synthetic Biology

Exploring the field of Synthetic Biology and how it unfolds as an engineering discipline.

As it has been stressed in the introduction, Synthetic Biology is a promiscuous discipline in the sense that it has, somehow, intricate relations with many other disciplines. Because of being so appealing and of having no clear boundaries, there are many scientists that are doing significantly different types of research and all claim to be doing Synthetic Biology. Hence, it is accepted that there are different branches or approaches under the reign of this broad discipline. However, as mentioned in the introduction chapter, in this PhD Thesis we just embrace one of such branches, the one that is engineering-oriented.

In order to properly set the stage for the further works we include, this first work presented here starts by tracing back the scientific roots of Synthetic Biology as well as its sources. Thereafter, it covers its contemporary history, the last 15 years, reviewing its main findings and the key discoveries and technologies that have shaped its evolution until nowadays, and provides a state-of-the-art of the topic. Finally, the last part of this work exposes the foundations that must sustain Synthetic Biology as an engineering discipline, it presents its strengths and weaknesses and critically discusses the factors that hinder its advance.

To write this review I have made a thorough documentation process. The work's inclusions criteria has been depending on their relevance to the field, without following a strict chronological order. Considering the size of the topic under revision, some key works may have laid unavoidably outside the final manuscript. Besides, the constant and rapid changing nature of this field represents an another challenge this review has to overcome not to lay soon out of fashion. Hence, this work is thought as an (static) frame of this field that captures the background of this discipline. Finally, the review deals with the future perspectives of the field,

especially regarding to its efforts to develop as an engineering discipline.

As a brief conclusion, I stress that Synthetic Biology still has much homework to do if it wants to become an engineering discipline. As such, it should permit the rational and straight-forward design and constructions of novel biological functions, devices and systems. The engineering principles used so far are a good starting point, however, still much has to be done to properly incorporate biological idiosyncrasy and understanding into such principles so that they can have success in helping to unlock the full potential of this emerging field.

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Review of Synthetic Biology: from its ancient roots to its engineering future.

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ABSTRACT

The goal of this critical review is to approach the field of Synthetic Biology from an historical and philosophical perspective. The idea is to briefly go back to its roots and follow them until the realization of what nowadays is known as Synthetic Biology. Considering that the term is in itself broad or even ambiguous, this critical review briefly discusses the main branches and definitions that have been developed under its umbrella, to later extensively focus in one of its most notorious branches, the engineering-oriented one. The main achievements and challenges that go in hand with this latest branch are presented following a relevance criteria in a non-strict chronological order. Finally, the principles upon which the marriage between biology and engineering is being built are exposed, together with a thorough discussion of the challenges and problems that arise from this encounter and that are yet to be overcome. This comprehensive review is aimed at properly contextualizing the field, critically looking both at its ancient roots and at its recent history within this century. At the same time, by discussing the principles that should govern this engineering branch of Synthetic Biology and the problems that hinder its advance, it shall contribute to create a necessary framework to move forward.

I. INTRODUCTION TO SYNTHETIC BIOLOGY

Given the plurality of definitions one can find about *Synthetic Biology* (SB) and the expectations, ambiguity, controversy and even refusal that are raised by these two words when placed together, the definition found in the *Wikipedia* might be a good place to start approaching the issue. It reads as follows:

[...] is an ***interdisciplinary*** *branch of biology and engineering, combining disciplines such as biotechnology, evolutionary biology, molecular biology, systems biology, biophysics, computer engineering, and genetic engineering. [...] is* ***designing*** *and* ***constructing*** *biological modules, biological systems, and biological machines for useful purposes.*

Retrieved from Wikipedia (February, 2016)

The first thing that pops up is that SB is an interdisciplinary field. It synergistically combines interests, methods, scope and knowledge from other disciplines, ranging from biology and chemistry to physics and engineering (**see Figure 1a**). Reading further, one sees that the definition is somehow similar to the one applied to Biotechnology also found in the *Wikipedia*, but with some key differences such as the presence of concepts like '*design*' and '*construction*'. Both of these concepts point out the fact that SB goes beyond Biotechnology in the combination of technology and living systems. Unlike in Biotechnology, SB not only seeks to modify organisms for a specific use but it rather looks for their *rational* design and construction from scratch with the aim of creating new biological modules and systems with different purposes.

Another thing that quickly draws the attention of any reader not familiar with the field is indeed the name. It is composed by the junction of two words, '*synthetic*' and '*biology*', that seem to have opposite meanings or to belong to very different worlds. When saying '*synthetic*' something man-made comes to our minds, while at the same time '*biology*' is related to nature, i.e. not man-made things (1) (see **Figure 1b**). The combination of both words might initially sound strange and surely contributes to the controversy and speculation that surrounds this field. However, two important aspects shall be highlighted after a deeper analysis of them: first, there is a branch of SB that is actually orientated towards the creation of life from scratch (i.e. man-made) and, in fact, the roots of the field could somehow be traced back to those investigating the origin of life, initially philosophers and later scientists; and second, from an etymological perspective, the word synthesis comes from the Ancient Greek *súnthesis* (i.e. a putting together, composition), which clearly sets the stage for the emergence of the combinative (building-up) engineering branch of the field that has garnered so much attention the last decade.

These two main branches are often respectively referred to as bottom-up and top-down, in the sense that the former pursues to discover the ingredients and the interactions rules needed for the appearance of life, while the second relies on subjects (at the top of the hierarchy) with a vision of the whole who actively and rationally design and build, either new forms of life or modify existing ones to create living applications

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†Javier Macía and Carlos Rodríguez-Caso equally contributed to this work.

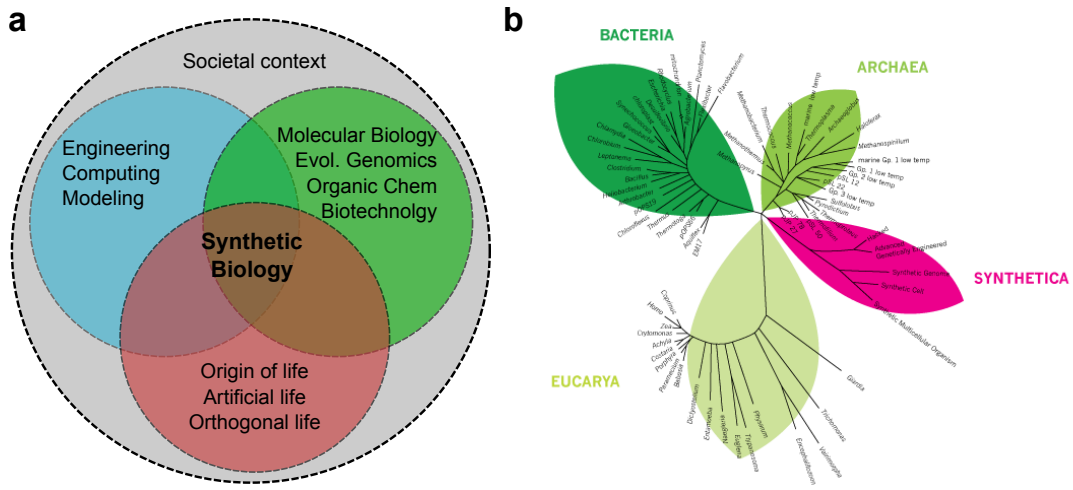

Figure 1. Synthetic Biology. (a) Synthetic Biology is an interdisciplinary field that is built and enriched thanks to the synergistic combination of knowledge and methods coming from several disciplines that span almost the whole scientific landscape. The dashed lines exemplify the fact that the field is always open to contributions of any other areas or disciplines *(Adapted from the European Science Foundation Website and Porcar and Pereto (1))*. **(b)** The new Tree of Life with the incorporation of a new branch of organisms that are the ones that result from the activity of Synthetic Biology, either because it creates them from scratch or it modifies the already existing ones (*Adapted from the front cover of the Book: Synthetic Biology - A Primer; figure made by Daisy Ginsberg*) (2).

(3, 4). However, it is worth noting here that this terminology may lead to a confusion, as the process of building-up new devices and systems from the combination of a given set of parts inherent to the engineering branch is also often referred to as being bottom-up (5). For the sake of clarity, we will consider here as bottom-up any process that departs from simpler parts and scales up to more complex systems.

I. I. Inspiration from philosophers and other thinkers

Regardless of which of both approaches we consider, there is a famous quote, especially among synthetic biologists, that is worth to bring up here. The author is a well-known physicist and Nobel laureate, Richard Feynman, which at the time of his death had written on his blackboard:

What I cannot create I do not understand.

Although for sure he was not thinking of SB when he wrote this sentence, it fairly illustrates the philosophy that drives the different branches within this field: when we try to build things -and we do- we need to have a prior knowledge of what we are doing but there is also a valuable process of gaining insight and understanding during the design and building processes. It is worth stressing that both failed attempts and mistakes contribute to that understanding.

The deep roots of the field go back further in time, especially if we consider the search for the origins of life. R. Descartes himself, in the year 1632 (posthumously published in 1664), conceptualized living bodies as mechanical machines (i.e. automata) in a view that, despite the differences and given all the (molecular) knowledge we currently have, set the stage for the engineering of cells in SB as if they where biological machines (6, 7). It might seem reasonable to think of cells as machines that can be (re)engineered following mechanistic principles. At the same time, it is highly arguable whether life can arise from a predefined top-down design or, whether there will always lack some 'ingredients', as discussed before in relation with the bottom-up approach (8, 9).

Following this line, and still in the philosophical arena, I. Kant discussed these ideas conceding that knowledge must follow mechanistic principles but arguing against the fact that organisms or living bodies can be explained only through such principles. He pointed to additional necessities such as the ones that nowadays are known as self-replication, autopoiesis and evolvability (7, 10, 11). These concepts somehow take inspiration from Gohete's idea of thinking of bodies (or cells, we shall add) in terms of actively self-driven systems (12). Although it is not the scope of this review to look for the origins of life and discuss it, we can undoubtedly ascertain that SB is a tool that can help us to shed light on these and many other questions. Furthermore, and now that we are dealing with the philosophical roots of the field, it is nonetheless significant that Kant himself expressed thoughts that are closely related to -and could be considered predecessors of- Feynman's idea quoted before when he said, approximately two centuries earlier:

Reason has insight only into what it itself produces according to its own plan.

These concepts and ideas exposed have inspired scientific researchers for a long time. But, *what is the connection of this big philosopher with SB?* Now, we are going to analyze the scientific field that is known as SB, conceived at the dawn of this century. More precisely, we are going to focus on its rational engineering branch. It is worth first paying attention to how scientists started to approach the field, to what it was

or what it meant initially to them and how those initial steps have brought us to the current *status* of the field.

I. II. Tracing back the scientific roots of SB

Going further in time and entering now into the scientific arena, it is at the beginning of the 20th century where we find the first explicit reference to SB. It is found in a book published by a French biophysicist, Stéphane Leduc, that is actually entitled *La Biologie Synthetique* (i.e. synthetic biology) (13). Leduc, together with another Mexican scientist of his time, Alfonso L. Herrera, thought that life could be created, and its underlying mechanisms understood, through a process of synthesis from the inanimate world. Thus, it is worth noting here that these initial references to SB are more related to the search for the origins of life and our capacity to synthesize it rather than to the engineering view.

Although they were not able to find the answers, they pioneered the search for the origins of life and probably coined, for the first time, the term synthetic biology (see evolution in the use of the words '*Synthetic Biology*' in **Figure 2**). This search continued over the years with many notable advances and brilliant scientists pushing forward the frontiers of our knowledge on the issue. Without going into detail and knowing that we might not include many scientists and their work, it is worth mentioning here some important contributions to the topic. For instance, the work of A. Oparin relating the chemical origin of life with the evolutionary theory, the experiments of Stanley Miller simulating the primitive prebiotic synthesis of organic molecules and the work of those working nowadays in protocell biology (9), among others. Nevertheless, the course of this early SB and its path to find the origins of life as well as to address other scientifically interesting issues started to change in the second part of the 20th century.

The work of François Jacob and Jacques Monod brings us closer to the definition of SB that considers the study of the principles of biological organisation and the manipulation of living systems from an engineering perspective. Jacob and Monod's seminal work about the *lac* operon in *E. coli* showed that gene regulatory circuits were able to command cell's responses to the environment (14). More details about transcriptional regulation in bacteria that were discovered the following years helped to reinforce and expand this vision (15). All this, added to the new revolutionary techniques that were developed during the 70s and 80 (e.g. the PCR and molecular cloning (16)), led to an expansion of genetic manipulation. This, in turn, fostered the the launch of a sub-field of Biotechnology that was called genetic engineering. This discipline is often seen as the most common ancestor of what we know today with the name of SB. It is worth noting that the term SB was already used at that time to refer to bacteria that had been engineered with the aid of the new DNA recombinant technologies developed within this sub-field (17).

The next decade -the 90s- there was a huge acceleration in the field of molecular biology thanks to new technologies that were being developed at that time such as automated DNA sequencing (18) and high-throughput techniques for measuring RNA, protein, lipids and other metabolites (19, 20, 21). Furthermore, the improvement of computing capacity and the development of new tools for modeling cellular

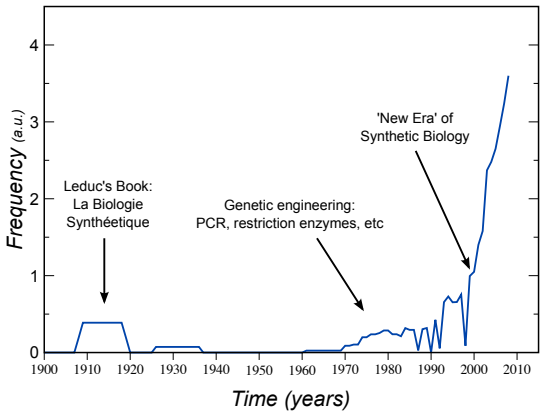

Figure 2. Historical evolution of Synthetic Biology. The frequency of appearance of the words "Synthetic Biology" in books written in English from 1500 to the present. These two words, that did not appear in a book until the 20th century, reflect somehow the historical evolution of the field: a first burst that correlates with the publication of Leduc's first book; then a second rise that seems to be related with development of genetic engineering and related techniques (e.g. PCR, restriction enzymes, etc); and, finally, the sharp increase that starts around the beginning of the 21st century with the appearence of the first Synthetic Biology papers (e.g. the *repressilator* and the *toggle-switch*) and continues growing until nowadays. These results were obtained using the search engine of Google Books and its Ngram Viewer, which charts frequencies of any set of strings using a yearly count of n-grams found in sources printed between 1500 and 2008. Frequency data displayed with arbitrary units.

processes were also important cornerstones to the field. Altogether coalesced into a new discipline aimed at the study of cellular networks at different levels and the integration of such information in what was, since then, known as Systems Biology (22, 23, 24). At this point, taking the genetic engineering field one leap forward to the rational manipulation of biological systems became a real possibility (25).

I. III. The new era of Synthetic Biology

It was not until the beginning of the 21st century that the name "Synthetic Biology" was coined again (see **Figure 2**), in this case in a talk given by Eric Kool at the annual meeting of the American Chemical Society in San Francisco (26). The idea behind the approach undertaken by Kool and colleagues (and that fell under the umbrella of SB) was to design and synthetize unnatural molecules inspired by those found in nature so that they that can be a substrate of Darwinian evolution and therefore function in living systems (27).

Although this might not be the most popular branch of SB it is still very relevant (28, 29). This branch also hosts another approach, known as the 'minimal living cell', that pursues the bottom-up creation of synthetic or semi-synthetic cells from scratch (29). The idea of these approaches is, on the one hand, to gain understanding and insight about how cells function (and why) through synthesis, and on the other hand, to answer the question of which is the minimum complexity (e.g. minimum number of genes, essential molecular components, reactions and conditions, etcetera) that is needed to support

life (30, 31) and to achieve a chemical understanding of life. For instance, the recent artificial synthesis of the minimal known bacterial genome -the J*CVI-syn3.0*- with 476 genes, less than the 525 of genes of *Mycoplasma genitalium*, might represent a hallmark of this approach (32). Notably, these issues are closely related to the goals that were pursued by the historical pioneers looking for the origins of life that were mentioned before.

Now is time to explore the other branch of the field, that is the one that very often draws more (public) attention and resources and, as mentioned before, is the one we put our spotlight on in this review: the **engineering** one. This branch took off when the engineering community met the molecular-biology *wetlab*. At that time, around the turn of millennium, the necessary ingredients for the emergence of this branch of SB were ready and the momentum was strong enough. A number of new technologies for manipulating and even editing the DNA at wish were available. There was a deeper knowledge than ever of the cellular processes and a map of the interactions taking place at different levels among the cell's vast catalogue of molecules. Moreover, lots of different computational tools for gathering and analyzing huge amounts of data, for its predictive treatment and even for the *in silico* modeling of genetic circuits began to be incorporated into the daily life of scientists within the field. Framed around Systems Biology, all this paved the way for the emergence of Synthetic Biology (33). More importantly, it set the groundwork for this engineering branch of SB that had recently gained publicity and that took off at the start of the century.

Hence, bearing all these in mind, the goal previously mentioned of a *rational* design and construction of biological systems with different purposes became gradually a real possibility. This engineer-oriented branch of SB, as S.A. Benner described in his seminal review of the field, "*seeks to extract from living systems interchangeable parts that might be tested, validated as construction units, and reassembled to create devices that might (or might not) have analogues in living systems*" (27). This branch of SB offers a bottom-up approach that is based on a continuously growing list of molecular 'parts' that can be combined and (re)assembled to rationally forward-engineer regulatory circuits in order to create new devices with an open horizon of possibilities.

II. THE ENGINEERING BRANCH OF SB

After the general overview covering the background and diversity of SB made so far, we shall now take an extensive tour through the history of this engineering branch of SB, since its inception until its present ongoing development. There are some good reviews on this topic (see refs (27, 34, 35, 36, 37), though these are focused either on a particular sub-topic (e.g. RNA Synthetic Biology) or on the whole field, covering different branches. However, the aim here is to make a broader tour through this engineering-oriented branch while highlighting the main achievements, the different technical improvements and the conceptual developments and applications that have gone hand by hand with the growth of the field. Furthermore, in the last sections, the different and various difficulties that have opposed resistance to the growth of SB are exposed. We discuss how these difficulties have contributed to shape the content and direction of this SB. They somehow embody some of the key aspects that must be dealt with if we are to push forward this field.

II. I. The proof-of-principle stage

SB took off this new century with the (model-based) design and construction of simple gene regulatory circuits, using *E.coli* as the workhorse of these circuits and pursuing proof-of-principle projects (38). For instance, L. Serrano and A. Becskei published in the year 2000 a paper in *Nature* where they showed how a one-gene circuit implementing a negative feedback loop was able to provide stability to the circuit (39) (**see Figure 3**). Moreover, larger circuits were also published in the same year and the years to come (40). In fact, in the same issue of *Nature* in the January of that year, two of the most famous or cited SB circuits were published, involving two and three genes.

In the first one, T. Gardner and colleagues constructed what was called a *toggle-switch* -a two-gene synthetic bistable circuit- and modeled and studied its behavior (41). This circuit was built by connecting two repressible promoters in a mutually inhibitory network and can be used as a bit of cellular memory with a variety of potential applications. In the other work, M.B. Elowitz and S. Leibler rationally designed and constructed an oscillating three-gene network called the *repressilator*, as it was built by connecting three transcriptional repressor systems (LacI, TetR and cI). This system showed periodic oscillations through the expression of a green fluorescent protein. This circuit can be used for the building of new and improved synthetic cellular behaviors and could also help to increase our understanding of the natural ones (42) (see **Figure 3)**.

Finally, it is also worth mentioning the work of R. Weiss and S. Basu presented just two years after, at the Fist Workshop of Non-silicon computation. They discussed the concept of using genetic circuits to create logic gates that, when properly combined, could precisely control gene function and, therefore, program cell's behavior (i.e. cellular computation) (43). This work, which pursues to mimic electric circuits and logic computation with biological circuits, set the stage for much of the work that has been done since then and contributed to reinforce the role of electrical engineering as as an inspiring source and model for SB.

All these circuits, nonetheless, were designed and built in order to display behavior at a single-cell level. One step further in the field was the achievement of coordinated behavior among groups of cells. Considering the fact that genetic circuits are inserted at a single-cell level, the need to find a way to communicate bacterial cells with one another quickly popped up (44, 45). In order to do so, R. Weiss and T. Knight, pioneers of the field, took advantage of a naturally occurring system -the quorum sensing system of *Vibrio fischeri*- that since then has become widely used in the field (see examples and reviews (46, 47, 48, 49, 50, 51)). This system is based on a number of genes and promoters that produce and sense organic molecules called lactones, which diffuse in and out of the cell allowing for a coordinated response behavior. Such a behavior depends upon reaching a critical density of population or *quorum*, which is captured by a certain threshold of lactone concentration.

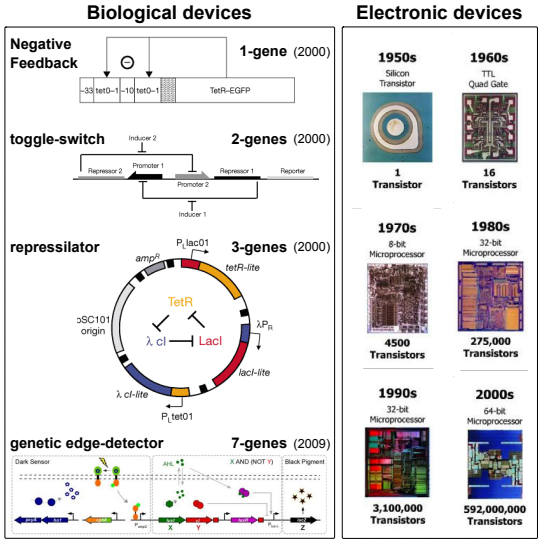

Figure 3. Comparision between electronic and biologic devices. The historical evolution of electronic devices and the amount of transistors they contained shows the exponential growth of this engineering field (e.g. from 1 in 1950 up to 4500 twenty years later). Regarding biological devices, the number of genes these comprise has grown from 1 to 7 in 10 years but despite the approach of the discipline's 20th anniversary, there are no signs of it making a big jump soon. This figure exemplifies the parallelism that is often drawn between the electrical engineering discipline and Synthetic Biology: ones serves as an inspiration to the other, though the challenges are not the same and, therefore, the pace of growth and capacity to deliver is different. (*Genetic circuits adapted from the original papers: (39, 40, 41, 42)*)

This new feature opened the door for going beyond single-cell behavior, thus paving the way for the synthetically induced multicellular behavior (52). One key example is the ability to program and control cell populations thanks to the coupling of cell survival and cell death by using quorum sensing molecules (lactones) and thus regulating the density of *E. coli* population (53). Another striking example of this, and that is of notable biological importance, is the achievement of collective pattern formation, of which the paper of S. Basu and colleagues is the very first example (54)

These works represent important achievements towards a better understanding of the design principles of nature and are key proof-of-principles with many potential applications. Seen from an engineering perspective, in contrast with the traditional one focused on description and characterization of the systems (e.g. cellular and molecular biology), this approach seeks to use, adapt and apply common engineering principles to work with living systems. However, and as mentioned before, researchers of the field also performed investigations that were more oriented towards the development of direct applications rather than to basic research issues such as the ones mentioned so far.

A good example of this that had a lot of media impact was the engineering of the Artemisin precursor pathway in *E. coli*. This achievement opened the door for massive production of this compound in a microbial host. This, in turn, might help to lower the compound's market price, as it is normally extracted from plants, from which low concentration yields are obtained. Hence, considering that Artemisin is used to treat a disease responsible for thousands of deaths (i.e. Malaria), especially in impoverished countries, this achievement and more of the kind that hopefully might come could have a major social importance (55). However, it is worth stressing that this latest example and others that often bear the SB label strictly speaking belong more to the Biotechnological realm rather than to the SB one.

This is because these application-oriented kind of projects are not based on the application of SB principles for the bottom-up building of devices that should ideally drive these approaches, but on a more thick-brush trial-error way focused only on achieving the desired output. In an ideal case, the boundaries that differentiate SB from Biotechnology (or Metabolic Engineering, as this sub-field is often called) should be easily identifiable (56). However, as SB boundaries are still not well defined, examples such as the Artemisin one are also often considered to be part of SB.

II. II. Beyond bacteria: exploring yeast and mammalian cells

Until now the examples explained take place in bacteria, which was, and still is, one of the widespread and most used organisms in the field. However, soon after the first steps of the field the engineering of biological systems ceased to be limited to the bacterial kingdom. For instance, L. Serrano and colleagues showed early on how a genetic switch could be implemented in eukaryotic cells thanks to a genetic network implementing a positive feedback that is able to convert a graded response into a binary one (57). Moreover, a few years later, efforts to produce Artemisin moved to yeast cells (58, 59) and other genetic architectures were also assayed and implemented in such cells (60) (see (61) for a good review on the topic).

Interestingly, a bit of memory similar to the toggle-switch already commented for bacteria was also implemented in mammalian cells just a few years later (62). Actually, we find earlier attempts to engineer genetic circuits also in mammalian cells perhaps in the work of M. Fusseneger and his group. Their work was being developed parallel to the work done in bacteria and yeast. In 2002 there was an interesting review that covered some of the approaches undertaken up until that moment to engineer artificial mammalian gene networks and that speculated about its possible applicability (63).

Two years after this review, for instance, they published a paper describing how to create 'BioLogic gates' that should enable logical control in mammalian cells (64). These transcription control modules are able to respond to several inputs in a logical way and, therefore, depending on how they are connected, they should permit a transcriptional control of such cells. Furthermore, they speculate that these versatile tools for gene expression regulation and for the creation of artificial gene regulatory networks could have a myriad of applications ranging from gene therapy and tissue engineering to biotechnology.

II. III. Cellular circuits inspired in electric circuits

Most of the work in the beginnings had to do with proof-of-principles related to genetic circuit's engineering (i.e. the creation of cellular circuits). In computing, systems that make complex decisions and perform a vast number of difficult tasks are a must. Likewise, in SB the idea is to create cellular circuits that should allow us to develop advanced applications and to create synthetic cells able to perform complex tasks, thus helping to make a big leap in what SB can deliver. However, as in electronics, more complex circuits implies larger circuits and, therefore, the need to scale-up designs. Sadly, as we shall see, to go from the proof-of-principle stage to a consolidation one in which designs are easily scaled-up is not an easy and direct step.

This particular approach was inspired by the background of those researchers that came from the field of electrical engineering. They were used to apply modeling approaches and combine them with all the technological tools for the construction and combination of electrical components. By doing so, they showed an ability to create complex electrical circuits that can perform a myriad of tasks with huge potential applications. Some of these engineers later tried their hand at the *wetlab*. They took advantage of all the knowledge currently available in the postgenomic era about the connectivity of genes and proteins as well as of the techniques developed for genetic engineering and all the modeling tools available at that time. The result was this sub-field directed towards the design and construction of genetic circuits inspired in electric ones (65, 66)

There are many examples of different circuits implementing several kinds of logic gates and all of them cannot be explained here, however, some of the ones developed in recent years should be, at least, mentioned. For instance, C.A. Voigt and colleagues combined NOR gates with quorum sensing molecules acting as wires and using different spatial configurations in order to achieve robust multicellular computation (67). Furthermore, in another work they were able to connect in layers several AND gates in a single cell. They showed how using multiple transcriptional activators and chaperon pairs large and integrated circuits in single cells could be obtained (68).

Moreover, parallel to Voigt's work, in that year and in the same issue, another strategy for achieving cellular computation with the help of cellular consortia was presented (69). The idea was to show how the wiring problem could be solved thanks to the use of cell consortia and a distributed output (70, 71). Besides, the appearance of a new method for DNA rearrangement based on bacteriophage recombinases (72, 73), made possible other new strategies for the creation of logic gates and other kinds of circuits such as those exhibiting memory (74, 75) or even logic gates able to amplify the signal (76).

Notably, most of the genetic circuits explained so far and that went in hand with the launch of the field were based on a DNA-transcriptional level. However, since the initial steps of the field other interesting levels of genetic regulation and molecular tools have been explored without leaving aside the aforementioned one, even including the combination of different of such levels (77, 78, 79).

RNA as a tool for CIRCUIT'S DESIGN

One important layer susceptible to engineering lies in the RNA world. In the last years there have been ascertained multiple and exciting new roles for RNA molecules besides its well-known role as the messenger of gene expression that connects transcription with translation in the central dogma (15). RNA, a molecule with a notable versatility, is able to interact with nucleic acids but also with proteins and even small molecules in its different forms and shapes (80, 81). Few years after the SB field appeared, researchers demonstrated the possibility of engineering these molecules in diverse ways to obtain novel functions benefiting from its particular characteristics.

One of the first works on the issue was done by F.J. Isaacs and colleagues in 2004: a riboregulator mechanism for *E.coli* (82). They showed how to engineer gene regulation with the addition a cis-repressive sequence to gene sequences in order to silence them by preventing ribosome attachment. Furthermore, they proved how to re-activate gene function through the expression of a trans-activation small non-coding RNA that structurally interfered with the repressive loop that was created by the cis-sequence. This example showed how a precise control of gene expression could be designed thanks to riboregulators created through the engineering and use of specific RNA-RNA interactions. Moreover, it served to open the door for its use as components for the synthetic circuits that were being developed within this new era of genetic engineering (36).

Furthermore, works such as the one led by C.D. Smolke showed how other types of riboregulators could be further engineered so that they could be regulated in a ligand-dependent manner by different cellular effectors. These riboregulators should in principle permit the regulation of the expression of any target transcript in response to any ligand. Called antiswitches, these allosteric riboregulators developed in *S. cerevisiae* should allow for the control of cellular behavior and genetic networks taking into account the cellular state as well as environmental stimuli (see **Figure 4a**) (83) .

Many more riboregulators and other RNA tools have been developed in recent years that allow better programming and control of cellular behaviors (84). And this has not happened only in bacterial and yeast cells but it has also been extended to mammalian systems (79). This RNA branch of SB is not the main object of study of this review, however, it is an ongoing part of the field that has been on the rise in the last few years. It still has several interesting challenges to face and holds a promising future that, among others, includes the interaction of RNA SB with nanotechnology (85).

PROTEINS as a tool for CIRCUIT'S DESIGN

Another level of action of SB that is not the transcriptional one or the post-transcriptional one just seen (RNA level) focuses on protein regulation (i.e. translational and post-translational). Although there are some examples that could fit in this protein SB level that are contemporary to the other levels already exposed these are more an exception than a rule. The inherent nature of proteins makes them a double-hedge sword for SB. On one hand, proteins hold great potential due to its versatility, inherent complexity, dynamics and diversity of interactions. Either already existing ones, newly synthesized ones or even networks of proteins, proteins

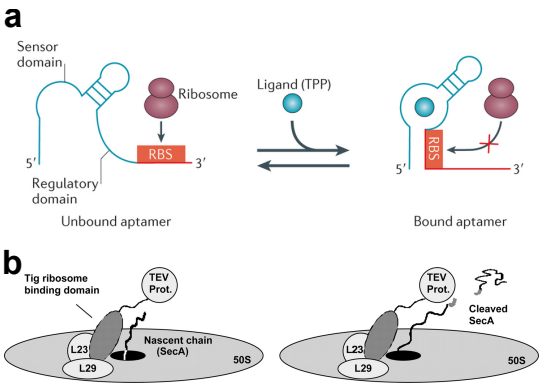

Figure 4. Other tools for synthetic circuit's design. (a) RNA. Example of a ligand-sensing synthetic riboswitch. It is found upstream of a given gene, regulating its expression upon the binding of a signal (small molecule or peptide) that triggers a conformational change and therefore changes the status of the riboswitch. In this example shown, a thiamine pyrophosphate (TPP)-responsive riboswitch binds to TPP and inhibits translation by blocking access from the ribosome to the RBS (*Adapted from Qi and Arkin (81)*). **(b) Proteins.** The tobacco etch virus (TEV) NIa protease is used due to its capacity to cut proteins at specific sites by recognizing a seven-residue consensus sequence. If proteins are engineered to contain this sequence, they are identified and cut by the TEV protease. In this case the TEV protease has been attached to the ribosome to increase its cutting efficiency and is able to cut *in vivo* a SecA protein that has been modified to include the target sequence (*Adapted from Henrichs et al. (86)*).

can act at different scales, both temporal and spatial, and elicit a myriad of responses through different architectures (87, 88, 89). On the other hand, however, these features make them reluctant to system engineering, which could explain why they still lag behind other system engineering such as gene regulatory networks (37). Engineering of proteins at a single level, or at most the engineering of two-proteins level, has been a field of great success and improvement in the last decade and even more. Yet the jump from the engineering of one or two proteins to a whole system or network of proteins entails much more difficulty (90).

One of the very first examples of protein engineering in SB took place in yeast: W.A. Lim and colleagues rewired a *Saccharomyces cerevisiae* pathway in order to discern the role of scaffold proteins in signaling pathways such as the MAP kinase pathway (91). It is worth noting, however, that some years later they made a leap forward: not only were they able to rewire the MAP kinase pathway with non-natural input-output properties, but they engineered the post-translational interactions of the pathway components in order to reshape the steady state and dynamic responses of this signal transduction cascade (92).

Notably, these are not the only works done on the subject. More work also on (synthetic) scaffolds has been done, for instance, in *E.coli* (93). Besides, protein SB has been directed towards the modification of protein's localization of a given pathway (i.e. make them colocalize) in order to improve yield production (94), to trigger spatial polarization (95) or even to allow specific processing of proteins *in vivo* (86) (see **Figure 4b**).

Although more examples of protein SB exist (96), all of them cannot be included as this is not the central goal of this paper. It is however worth stressing that most of the examples that belong to post-translational circuits are still at the very proof-of-concept stage. The main goal would be to be able to design and create protein circuits in a predictable manner, similar to what is intended for genetic transcriptional or post-transcriptional circuits (37). However, the methods and knowledge needed for such a purpose are still in its early infancy, though the field is moving forward. Finally, it is worth stressing that the creation of circuits including combinations of the different levels of action that have been mentioned above could be of great potential and help to push the frontiers of the field even further.

II. IV. Applications of SB

A lot of work on direct applications of SB has also been done in the last years with many notable successes. This work includes exploring and developing applications for a number of different areas, ranging from the biomedical to the industrial ones, as we are going to explore in the following subsections.

BIOMEDICAL applications

One of the areas in which more efforts and resources have been invested is the biomedical one. The idea is that SB can be a powerful tool to address several aspects related to human health. Through different mechanisms and strategies, SB can be useful in the study of disease mechanisms; drug development at different stages (e.g. discovery, production and delivery); and diseases prevention (e.g. vaccines) and treatment (e.g. cancer and infections) (97, 98, 99). A number of works supporting these statements exist, some of them are going to be highlighted here as examples.

Thanks to its building-up strategy and to the tools it provides, SB offers a good platform for a number of things. Among these we find the identification of the mechanisms underlying diseases such as agammaglobulinemia (100) or SARS (101) as well as of the sequence of events leading to a particular pathologic phenotype (102). It is also a powerful tool for several drug related issues: in drug discovery (i), helping to systematically screen and identify potential molecules that could serve as alternative antibiotics (103); in drug production (ii), as in the above mentioned Artemisin case (59); in vaccine development (iii), helping, for instance, in antigen presentation while avoiding the risk of infection by attenuated pathogens (104); in drug delivery systems (iv), taking advantage of a patient's microbiome, engineering it so that it serves medical purposes (e.g. manufacturing and secreting interesting molecules for the treatment and prevention of diseases such as diabetis (105), HIV (106) and other immune diseases (107)); and, finally, also for disease prevention and treatment (v) (99).

Regarding this last point, different strategies are worth highlighting. One is the use of bacteriophages that target antibiotic resistant bacteria and make them vulnerable again to antibiotic's action, for example by disrupting biofilm's integrity or by acting as antibiotics adjuvants (108, 109).

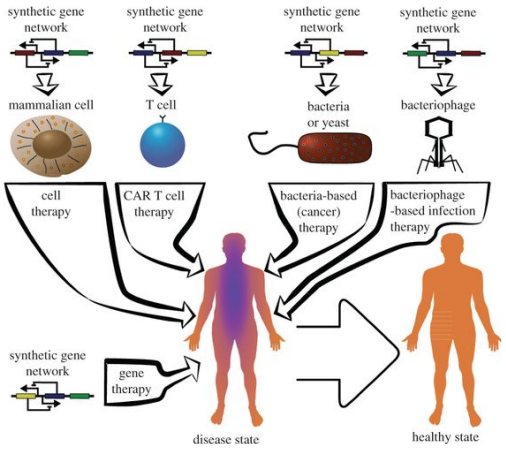

Figure 5. Synthetic Biology for biomedical applications. SB helps the healthcare spectrum from diagnosis of diseases, to drug screening, drug biomanufacturing and to therapy. Here we show different approaches that already exists for the treatment of several diseases (e.g. cancer, infections, etc.) by engineering different cells (i.e. cell therapy): mammalian cells, T-cells, bacteria or yeast, bacteriophages and even gene therapy are possible options (*Adapted from Kis et al.* (79))

Another important one is the cell-therapy approach (i.e. the introduction of engineered cells into the body to treat a disease), remarkable examples of which are the use of engineered T cells to combat cancer cells (110) and of other engineered cells able to control urate homeostasis to prevent and fight disorders like the tumor lysis syndrome and gout (111). The possibilities are vast and there's room for more new strategies and other innovations (see **Figure 5**).

INDUSTRY-related and OTHER applications

SB applications can also be oriented towards solving other very different, yet very important, human-related matters. Framed in the industry world, these applications aim to provide more efficient solutions, and if possible less environmentally dangerous, to issues as diverse as biofuel, biomaterials and pharmaceuticals production (see reviews (112, 113, 114, 115)). The driving idea is to use cells as small, yet powerful, factories. Their genome might be modified for this purpose or even full synthetic pathways (circuits) can be embedded in them so that, when cultured under the appropriate conditions, they may produce great amounts of a desired compound (e.g. artemisin, biodiesel, and so on) (59, 116, 117). Notably, as commented before, this approach is often referred to as metabolic engineering (56).

Cell's metabolism has been forward-engineered using for example heterologous enzymes in order to produce isobutanol, biodiesel, gasoline and even bioplastics (117, 118, 119, 120, 121). Furthermore, different mechanisms for regulating and controlling the dynamics of the pathways in response to different conditions, such as key intermediate metabolites or environmental factors, have been successfully engineered and implemented in different cells (122, 123, 124). The tandem of SB and the industry is expected to have a short-term direct impact on society. Thanks to the continuously growing achievements provided by SB basic research it can expand its scope, methods and goals. Furthermore, the new technological innovations that are appearing every day and the obvious economic benefits that may provide, hopefully environmentally friendly, can also foster its progress.

III. ENGINEERING MEETS BIOLOGY AND VICEVERSA

After this general overview of the field, we are now in position to critically review the association between engineering and biology, to remind us upon what it is based and to expose and discuss its weaknesses and how to tackle them. Whereas biology is aimed at the study of living things, when one thinks of engineering, either in an abstract way or in any of its different engineering disciplines, one intuitively knows that it has to do with the way in which diverse kinds of scientific knowledge are combined to design and build any useful application for human purposes. However, with SB it might be the first time that engineering tries to apply biological knowledge to "invent, design and build" biological or living applications.

Until the renaissance of SB this century (13), engineering applied knowledge coming from mathematics, physics and engineering disciplines in order to build upon inanimate things or materials. Thus, depending on the knowledge and methods used and the purposes followed, there have been created and developed during the last centuries different engineering disciplines and sub-disciplines, such as the electrical, chemical, mechanical and civil, to name the main ones. Regarding engineering living systems, it is worth stressing that the first steps towards the combination of nature and technology were performed by biotechnologists a long time ago. However, as commented in the beginning of this review, it is arguable whether it is not since the recent birth of SB - and its engineering branch- that the integration of biological knowledge, vision and tools into the engineering family has truly been tried (125, 126, 127).

Hence, this type of SB implies engineering and biology working together. In order to be successful, the marriage between science -biology- and engineering has to nurture both pillars that support it. As R. Brent pointed out, *"this intersection of science and engineering can spur the development of both"*. Additionally, he emphasized that the practitioners need to be "*honest with one another about the limits of their abilities*" (128). The combination of the investigative nature of science with the constructive nature of engineering is nothing but the key underlying the power of SB (129, 130).

However, although SB is quickly approaching two decades of life, as an engineering discipline it is still in its *infancy* (131, 132). In one of its co-founders words, T. Gardner, "*the promise of synthetic biology lies in its engineering roots*", however, "*its founding premise has not yet been validated*" (133, 134). As a founding premise, Gardner refers to the idea "*that standardization and abstraction of biological components will unlock the full potential of biological engineering*" (133). In other words, the application of engineering principles (e.g. standardization and abstraction) to biological systems lies at

the core of SB as an engineering discipline. It is indeed this fact what should foster its full development and help it to deliver the enormous amount of things that it is expected to create (131, 133).

Nevertheless, to "engineer life", as Phillip Ball has called it (135), it is an extremely difficult task precisely due to life's complex nature. Or in the words of A.A. Cheng and T.K. Lu (exchanging 'life' for 'biology'): "*compared with other substrates, biology poses a unique set of engineering challenges resulting from an incomplete understanding of natural biological systems and tools for manipulating them*" (136). SB, compared to other engineering disciplines, is still far from having the mechanistic insights that are needed for a proper, reliable, robust, predictive and rational forward-engineering of biological systems (34).

This section will be divided in two main parts: first, an explanation of the efforts undertaken until now in order to establish and apply or adapt (new) engineering principles for SB; and second, an overview of the many constraints, challenges and problems that arise from this encounter between biology and engineering.

III. I. Engineering principles for SB

In the first years of SB, practitioners were mainly working intuitively, applying the concepts and experience they had from their previous fields. Those coming from molecular biology, for instance, were more used to proceeding in an erratic trial-error way. Indeed, in this way the field delivered its first results, and still mainly does nowadays. On the other hand, there were engineers learning molecular biology concepts and methods. Due to its background, the latter ones were more prone to looking for general principles of design and construction and trying to apply them in order to create SB products.

In any case, the idea of creating, developing or directly applying common engineering principles to biological systems was already present in the first steps of this field. However, it was not until five years after its birth when a seminal paper set the ground for the growth of this idea. Back in 2005, D. Endy published in *Nature* his review entitled '*Foundations for engineering biology*' (137). In this work, having on his side the perspective given by the years of work undertaken by the community, with its successes and failures, he proposed to adapt a set of principles borrowed from other engineering disciplines in order to facilitate a quick and reliable engineering of biological systems. The idea was to try to overcome or to deal with the inherent complexity of living systems to make possible the routine engineering of them.

The first three main principles he proposed were *standardization*, *decoupling* or *modularity* and *abstraction*. Soon after this work, others contributed reinforcing these ideas but also warned about the possible difficulties yet to be faced and solved in this endeavor, namely, *what makes synthetic biology different* (138). This vague sentence encapsulates several of the problems related to the fact that we are dealing with living systems, which are noisy, variable, subject to evolution and much more complex than the inanimate ones, among other things (138, 139). Now, before exploring in deep detail these problems, we are going to expose and discuss how people have tried to apply these principles to living systems.

STANDARDIZATION

Standardization is fundamentally a technical enterprise, the result of scientific and technological activity. The main scientific function of standardization is the collaborative production and dissemination of technical knowledge. Standardization is achieved through the development and implementation of *technical standards* within a given scientific or technological area of knowledge (140, 141). It helps to maximize compatibility, interoperability, safety, repeatability or quality, among other things. From an historical perspective, standardization in industry and commerce acquired importance with the onset of the Industrial Revolution, and it was actually the scientific standardization process that paved the way for the industrial standardization one (142, 143).

It is worth noting that engineering practices, independently of the sub-discipline in which one is working, take place within a community. Although this word is not explicitly found in the explanation given above, it is implicit. In fact, words like '*collaborative*', '*interoperability*', '*compatibility*' and '*interchangeable*' point out to the need of sharing within this community and to do so in a '*uniform*' and agreed way. This last thing is reflected in the establishment of the so-called technical standards that are supposed to be followed by the whole community. These standards are an established norm or requirement related to technical systems. They are usually a formal document that establishes uniform engineering or technical criteria, methods, processes and practices.

It is also worth mentioning the relation that was established between the onset of the Industrial Revolution and the implementation of technical standards in engineering, at that time mostly mechanical. A parallelism could be drawn between the Industrial Revolution and the Revolution that Synthetic Biology pretends to accomplish. In order to reach that goal we should be able to develop high-quality standards and spread them along the whole community. By using such standards, synthetic biologists around the world could collaborate in projects and share their findings and products so that people could build and construct upon things done by others working in the field with relative ease and somehow speaking the same biological-engineering 'language', and so each project should not be started from zero.

In his paper, D. Endy stressed the need to "*develop formal, widely used standards for most classes of basic biological functions (for example, promoter activity), experimental measurements (for example, protein concentrations) and system operation (for example, genetic background, media, growth rate, environmental conditions, and so on)*" (137). Hence, standards should not be developed only for the definition, description and characterization of biological functions or '*parts*', but also for the way in which such parts and the construct they create should be assembled (144) and measured (145) and for the conditions that support these genetic devices and the overall system's operating conditions (137). All this information should allow for a more efficient, predictable and design-driven engineering discipline around living systems (146).

Interestingly, insisting in the analogies with the electrical engineering field (see **Figure 3b**), the information comprised in the Pocket Data Book about logic electrical components and circuits represents a paradigmatic example of a good standardization process (147). In the same way, there are some ongoing initiatives to bring standardization to SB that emerged from the engineering community. The most notable one was developed at the MIT soon after the first steps of the field. The creation of the 'Registry of Standard Biological Parts' (*RSBP*) was meant to establish a repository in which to digitally catalogue and physically store an ever-increasing list of biological parts, either of new creation or already existing ones (148). Furthermore, they provided the 'Biobrick' standard format (149) to facilitate an easy, step-wise and methodical assembly of these parts into larger circuits, which at the same time could be incorporated in the registry (145, 150). The years have revealed that this initiative has some weaknesses that should be addressed if it is to be welcomed as the new 'Standard Book' for SB (151).

Along with the BioBrick format, other assembly methods and standards have also been developed such as the Gibson Assembly (152) and the Golden Gate (153). Moreover, as the price, speed and availability of DNA synthesis has notably diminished in the recent years (154), this technology can ultimately become a substitute of DNA assembly techniques for the creation of large pieces or constructs of DNA.

It is worth stressing that these part repositories need to contain detailed information and specifications of the parts they contain in order to allow for their proper use, either in the way they are provided or for the purpose of combining them in larger and more complex circuits. Such information regarding SB components is usually collected in Datasheets. One of the very first examples of such Datasheets for SB was provided by B. Canton and colleagues (145) In their work, as a case of study, they offer the information and characterization of a genetic regulable device made using parts from the *RSBP*. The Datasheet contains extensive details of the device composition and performance, both static and dynamic, as well as of the system requirements and its operating conditions. It includes, for instance, a transfer function -a concept that is again borrowed from electronics- that relates the different input concentrations with its correspondent output levels (48, 155, 156).

It is worth noting that, besides individual parts libraries have also been created of different part types such as promoters or RBSs with different intensities, which have also been added to the *RSBP* and to other repositories (157, 158, 159). This should allow, for instance, a finer tuning of the circuit behavior as the parts used can be chosen from a vast catalogue where a wide range of a given characteristics can be found (e.g. promoters strength). In this line, the *RSBP* is not alone, but other initiatives like the 'International Open Facility Advancing Biotechnology' (*BIOFAB*) that wish to expand and improve the use of standards in SB and other quantitative approaches have emerged (160, 161, 162). However, knowing these quantitative characteristics requires of a proper and intensive *characterization* of all the parts incorporated into the registry and its qualitative and quantitative *validation*, which is not often the case. In fact, this is actually one of the unsolved matters for standardization in SB.

ABSTRACTION

Whereas standardization enables SB practitioners to work together, to speak the same engineering 'language' and build-up collectively upon what is done by others, *abstraction* is needed because it helps us to deal with and manage the complexity of the systems we want to engineer. Regardless of their nature, whether biological or technological, many systems exhibit such amount of complexity, such richness, that it becomes highly difficult for a single person to deeply comprehend all the necessary details of its different composing 'parts' at all levels. Abstraction is the process of picking out the essential features of objects and procedures, it means representing its common characteristics without including unnecessary background explanations and details (163, 164). It is, therefore, a powerful tool that should allow us to overcome our limited capacity to manage increasingly complex systems and to permit us to simplify the analysis of such a system and its design. In other words, it is like encapsulating the information of different levels and elements into grey boxes that require little information for its use in larger systems, thus abstracting away unneeded levels of detail (165).

It is worth stressing here that the concept of abstraction is intimately linked with the concept of hierarchy. As systems grow, both in size and complexity, the need emerges to organize such systems into different levels of information. Independently of whether we are talking about a computer or about a cell, hardly anybody is able to understand in full detail the different levels of complexity in which these systems are usually separated. That is, from the basic levels determined by the laws of chemistry and physics up to the high levels of organization of cellular or electronic components. And even more difficult than having a 'light' and superficial understanding is to be able to act upon these systems in detail at all of such levels in order to modify them.

Hence, this is the reason behind the need for *abstraction hierarchy* that allows us to organize information across several layers or levels and reduce it to a level of complexity that is manageable for anyone (**see Figure 6**) (137, 138, 139). This should allow people to work at any independent level of the hierarchy without the need to pay attention to what happens at the other levels, either lower or higher. Thanks to abstraction hierarchies, systems can be managed with little or limited exchange of information between the different levels. This should permit an abstraction of the elements composing the system to a somehow brief description based on key functions and requirements that are needed for a proper managing of the system they compose (146). When talking about engineering biology we might draw an abstraction hierarchy like the one that follows: from DNA to parts, to devices, to synthetic cells.

Interestingly, abstraction might be linked with the third principle, decoupling or modularity: as we shall see, given that the abstraction hierarchy works properly we then can talk about composability and functional modules in SB (132). Abstraction hierarchies become an organizational prerequisite for the modular combination of parts into more complex systems (139). They are, therefore, tied to characterization and modularity: they help us to parse biological complexity into more easily understood parts, modules or devices (166) and allow for the decomposition of a system into such basic functional parts (138).

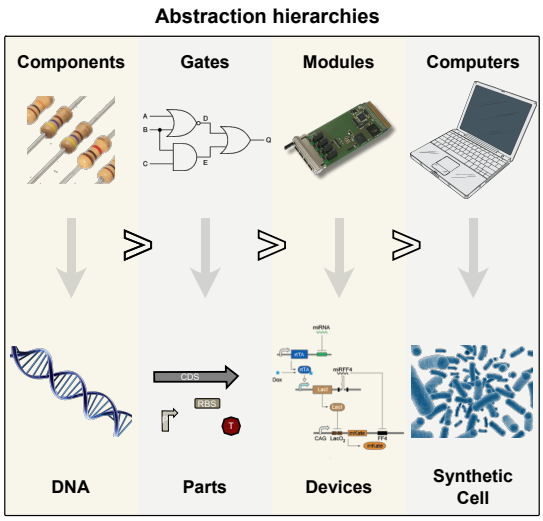

Figure 6. Abstraction hierarchies. Another parallelism with -or inspiration from- the electronic world. In electronics, there exist abstraction hierarchies that allow engineers to work at different levels or layers without the needing to have knowledge or a full view of the whole process, from the smaller electronic components to the full computer for example. In SB the idea is the same, the lowest level to work with with is the DNA, but one can climb through the hierarchy to work with genetic parts, devices and even synthetic cells or systems. If the principle of abstraction and the other engineering principles work as expected, one should be able to work at one given layer with little or no information of the other ones and yet the result should ideally be the one predicted (137).

DECOUPLING or MODULARITY

This later statement leads us to the third principle, decoupling, or what later was called or renamed by others as modularity (137, 139). In Endy's words: "*Decoupling is the idea that it is useful to separate a complicated problem into many simpler problems that can be worked on independently, such that the resulting work can eventually be combined to produce a functioning whole*".

One example of decoupling that is a hallmark of all true engineering disciplines is the separation of the design process from the actual fabrication of the components or systems that have been designed (139). This is done, in accordance with the definition just quoted, in order to facilitate the processes of design and fabrication. It should allow both processes to be worked independently from one another (i.e. by different experts with specific skills, knowledge and methodologies), while providing enough overlay or guidance so that when they are combined or put together the result is successful and predictable.

Notably, modularity is also understood as a property of those systems that can be deconstructed or decomposed to individual sub-parts. In a system where modularity applies perfectly, each of the sub-parts or modules should be able to perform its specific tasks independently from the other modules composing the system (165). Hence, it is worth noting that modularity between components allows for a building-up approach in which larger and more complex systems may result due to the many potential combinations among the different preexisting modules.

The concept of modularity lies, therefore, at the heart of SB. In classical engineering disciplines, modularity allows the insulation of interacting sub-parts from each other and make them interchangeable (138). Notably, modularity is closely related to another concept, *orthogonality*, which means that the different modules can be treated separately and are independent of, or irrelevant to, each other. This implies that in a perfectly modular system, all the orthogonal parts composing it may perform their function as expected or defined without any interference, either from other parts or the context, unless designed to do so. Hence, if a given system is modular one may be able to predict its behavior from the known behavior of its constituent parts and no unintended change should be expected upon interconnection of new parts or modules (167).

However, this kind of modularity, so complete, '*pure*' and useful in other engineering disciplines is hardly achieved in biological systems (25). This relative lack of modularity, at least the one achieved in SB until now, makes it difficult to forward engineer these kind of systems (165). Importantly, as we shall see later, cellular context might affect modularity as well as the other principles. Intracellular, intercellular and extracellular conditions have to be always taken into account as they might interfere with the modules themselves or with their connections (e.g. signal cross-talk) (129).

III. II. Problems, constraints & challenges for engineering in SB

The launch of SB came with great hype and expectation. However, fifteen years later, the field had not delivered what it was expected to. Despite all the efforts and money invested in turning SB into a true engineering discipline, one able to produce biological devices with reliability and relative ease, the design and construction processes are still tedious, unreliable and *ad hoc*. It is now worth reviewing which are the main constraints that limit the potential of the field, the challenges that are being faced and the problems that need to be addressed.

Prerequisite: BIOLOGICAL UNDERSTANDING

Besides these above-mentioned principles there is one key prerequisite to get SB closer to other engineering disciplines: the vital need for improving our *understanding* of biological systems, especially at cellular and molecular levels. Unlike most physical systems on which engineering is daily developed, with regard to living systems we are still far from having a complete understanding of them, or at least one enough to allow us to deal with them in a successful way similar to what happens in other engineering disciplines. Currently, it is difficult to reliably design novel SB applications or redesign existing ones; to easily and robustly predict the outcome of our actions upon biological systems; and to anticipate the different and diverse situations one may have while doing SB (8, 168, 169, 170).

Biological systems have unique features and differential traits that represent a new and hard challenge for engineering. If we are to transit from the current *ad hoc*, trial-error and roughly predictable pseudo-engineering SB to a truly engineering discipline performed upon living systems we should take into account and incorporate the biological *milieu* -the cellular context. For this to happen, not only do we have to increase our understanding of the systems we are dealing with, i.e. biological systems, but also to adjust and adapt our principles of design and construction and our methods to them.

Our in depth knowledge of biological systems at the molecular level has grown exponentially in the last decades and we are now close to having a full list of the molecules that take part in cellular processes and its interactions. However, we are still far from knowing how they work as a system and perform all the biological functions that are required (166). Similarly, we can now read and even synthetize DNA and RNA sequences without much trouble but it remains a big issue to predict the outcome of more than tiny changes on them (15, 18, 154). At the protein level, for instance, it is easy to obtain the amino acid sequence of a given protein and to find similar proteins. However, it is almost impossible to predict its function and how it will fold in a general way from the information of its sequence, and it is even harder to guess with whom it is going to interact and predict the consequences of such interactions (171, 172).

It is worth remembering here that after the disclosure of the results of the Human Genome Project (173), the general public -and even some scientists- thought that thereafter we should be able to rationally, easily and directly reveal the main questions that were until them resisting our attempts. However, it soon become clear that having a list of items or a book with the instructions is clearly different from being able to understand it. Furthermore, as we have already mentioned, at a cellular and molecular level, to foresee the outcome of our actions upon the system based on the information uncovered was far from straight-forward. Actually, another big collective scientific project that could be somehow considered its successor, the ENCODE project (174), has recently left it clear that we still have much to learn about this book and how to interpret it (175).

The goal of this review is also to contribute to the efforts devoted within the SB community to solve this and the other problems that hamper the advance of SB. As we are going to see next, besides our notable lack of knowledge about biological systems, there are other problems, numerous and diverse, that synthetic biologists must overcome and are often somehow related with this prerequisite just exposed.

Other PROBLEMS and CHALLENGES

It was back in 2010, ten years after the launch of the field, when R. Kwok published her notorious paper in which she pointed out the main challenges and problems that were hindering or preventing the expansion and maturation of the field (176). In fact, some of the issues she collected in that paper had already been pointed out by others scientists a few years before. For instance, Endy himself already mentioned some of the troubles that must be dealt with when engineering biological systems, namely: inherent biological complexity, spontaneous physical variation of biological system's behavior and evolution. All these things without taking into account the limited manipulation capacity of these systems we had and still have, though now at a lower degree (137).

Kwok summarized these and other difficulties in five points, or quoting her, '*five hard truths*', that SB has to face and hopefully overcome. First, there is the need to properly and fully define and characterize most of the biological parts (see, for instance, initiatives like the *RSBP* or the *BIOFAB* already commented (148, 160)). Second, the unpredictability of the circuitry is high: although parts or modules might be known and properly described, when they are put together their combination too frequently does not function as expected. Third, the complexity is unwieldy or, in other words, to scale-up SB products implies an exponential growth of the complexity associated with such designs. Fourth, the incompatibility of many parts, which refers to the relation between the genetic circuits and its hosts and the environment that is against a sort of 'universality' that would be desirable for SB. And fifth, is the inherent variability of biological systems: noise or random fluctuations, mutations and other variations hinder the predictive capacity of SB (129, 137, 138, 139, 176). Independently on how you group all these factors, either in five points or in other ways, it is clear that there exist serious difficulties to the routine rational engineering that would be desirable.

Actually, it is worth mentioning here that a very important player related to failed attempts in SB designs is sometimes referred to as '*the context*', a concept that comprises a number of different factors. S. Cardinale and A.P. Arkin broadly reviewed these issues and discussed them in a paper published in 2012, *Contextualizing context for synthetic biology - identifying causes of failure of synthetic biological systems* (177). Among environmental factors influencing SB devices they cite, for instance, fluctuations of physical variables, such as temperature and osmolarity, and dynamical changes in population density, diversity and interaction. They also emphasize that the prominent role of all the processes within the cell that depend on hosts properties via direct and indirect interactions with its cellular resources, machinery and all the components -and its concentrations- that are present within the cellular milieu. Finally, they discuss the compositional context that refers to the genetic elements that compose the genetic devices and its dependence upon the surrounding sequences.

When designing and building-up SB genetic devices we must not forget all these issues. If we are to pursue a straightforward and rational engineering of such systems one cannot ignore the unique features of biological systems ranging, for instance, from their capacity to evolve to the context in which they are involved (129, 178). Until nowadays, the predictability of SB designs is in question, as too many often unexpected factors or issues not properly taken into account are responsible of the common failures of SB designs. Ideally, SB should be context-independent or at least, the dependencies, if not possibly avoided should be adequately described, characterized and incorporated into the design process.

For instance, we can consider several modules that have to be combined to create a given system. These modules have to be, ideally, independent of each other and of the context. If so, the prior knowledge of such modules should allow to easily infer or predict the behavior of the system when they are combined. However, the obtained behavior too often greatly

differs from the one predicted and failure of the devices is the most common outcome. As this situation usually appears linked to the design and construction of increasingly complex devices comprising several modules, *metabolic load*, usually combined with cell growth effects, is pointed out as one of the usual suspects behind these failures (179, 180, 181).

Larger constructs made of more modules logically imply the consumption of more cellular resources. Considering that these are not unlimited, competition for the limited cellular resources has been suggested as a possible source behind failures in SB. In fact, global coupling among the different independent modules or parts, as well as with host genes, is thought to happen in SB due to the sharing of the limited pool of resources (177, 182). In other words, emergent and unexpected non-independencies (or interactions), either direct or indirect, may arise when combining the *a priori* independent and orthogonal modules (132). For instance, D. DelVecchio and colleagues coined the concept of retroactivity to give name to a similar situation in which the activity of downstream modules is unexpectedly affected by the behavior of upstream modules despite the fact they were supposed to be independent or act independently of each other (167).

One wonders how these and other *unexpected*, *unpredicted* or *non-regulated* interactions should be dealt with in order to improve the output and success of SB. Considering the need for a notable increase of our understanding of biological systems that has been broadly discussed, important efforts should be devoted to shed as much light as possible on these and other possibly relevant issues if we want to foster the progress of this discipline and push it to its further horizon.

IV. CONCLUSION

SB, still in its infancy?

So far, we have made a broad tour of SB. We have looked for its ancestors and traced its roots until the modern launch of the field. Later on we have followed its development and diversification and highlighted its main achievements until nowadays. As we have seen, SB encompasses many different disciplines, views and approaches and is fed by continuously developing technologies and new knowledge being uncovered. Such branches, approaches or ramifications coexisting under the umbrella of SB have also been slightly summarized here.

After the initial years, the field underwent a big expansion: every day more and more scientists got engaged in this exciting endeavor and hence the community grew rapidly and became even more international. This, coupled with the appearance of new technologies and the decreasing price of the existing ones (e.g. DNA synthesis and sequencing), contributed to an increase in the pace and scale of the scientific production brought by SB. The evolution of the field during these first 15 years has not been constant but it has had periods of accelerated growth or bursts of progress and others of more quiet stagnation. Among the factors that are responsible for these changes in pace and scale of SB there are those related to new technological discoveries or even those related to innovations provided by other scientific fields.

One nice example of this, which is lately revolutionizing SB in a way similar to what DNA sequencing and synthesis did some years ago (183), is the CRISPR-Cas9 system for genome editing (184, 185, 186). This system is a new tool that allows full genome editing, both of host genomes and of the synthetic circuits embedded in them. This method has successfully been implemented in bacteria, yeast and mammalian cells (187, 188, 189). Furthermore, this system -or modifications of it- not only permits genome editing but has also proved useful for regulating and targeting genomes (186). We cannot know yet what is going to be its impact on the field but a sure guess would be that it is going to act as a catalyst if it is not doing so yet.

At this point, this whole background should have allowed us to properly frame this SB that is mainly at the crossroads of *biology* and *engineering*, but that also integrates scientists, methodology and knowledge coming from very different and interesting disciplines or fields (34). Furthermore, we have also seen and discussed how this interaction between engineering and biology has been, and still is being, developed. We have exposed the pillars or principles that sustain it, with its strengths and potential, without forgetting its weaknesses. We have also explored the current perimeter of this field and which are the conceptual and technical issues that prevent its widening, the several problems and constrains that exist and the present challenges that have yet to be conquered.

This altogether draws a clear picture of the situation of SB for those that want it to be as fruitful and powerful as other engineering disciplines and aspire to see SB within the club of the other engineering disciplines: although it holds great potential, the field is still in its infancy. It has transited from an early infancy, represented by the first proof-of-principle circuits and devices, to a later one, exemplified by bigger circuits and an undeniable number of different applications and improvements. Nevertheless, it is now somehow stuck in this stage, unable to move forward, to cross a border and consolidate itself into a new stage in which routine and straight-forward design and construction of increasingly complex synthetic biology devices and systems should be the daily menu (130, 170).

Future perspectives

There is still a lot of work ahead. For this to be achieved, we should direct much more efforts and resources towards firmly establishing the principles that must govern this engineering discipline. We should move our attention from the fancy and good-selling projects that are far from this goal to those projects or studies that try to consolidate the bases or foundations of this engineering discipline.

Bearing all this in mind, we think that to do so requires better theorico-conceptual frameworks -experimentally backed-up- that have not yet been properly and extensively established. In an ideal scenario this should help to scale-up SB designs in a way similar to what happened in the beginnings of electronics, as it has been explained before. The jump from the current time-consuming and trial-error practices to ones much more predictable, reliable and robust is one of the main goals the field has yet to conquer. Reaching this cornerstone would unlock a huge amount of possibilities and perspectives for the SB community and society in general. If we do not want SB to become a giant with feet of clay but

a solid engineering-like discipline, we must help to build firm and strong pillars to sustain it.

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REFERENCES

1. Porcar, M. and Pereto, J. (2014) Synthetic Biology. From IGEM to the Artificial Cell, Springer, .
2. Baldwin, G., Bayer, T., Dickinson, R., Ellis, T., Freemont, P. S., Kitney, R. S., Polizzi, K., and Stan, G.-B. (2012) Synthetic Biology - a primer, Imperial College Press, .
3. Forlin, M., Lentini, R., and Mansy, S. S. (2012) Cellular imitations. *Current Opinion in Chemical Biology,* **16**(5-6), 586–592.
4. Voigt, C. a. (2006) Genetic parts to program bacteria. *Current opinion in biotechnology,* **17**(5), 548–57.
5. Nandagopal, N. and Elowitz, M. B. (2011) Synthetic biology: integrated gene circuits. *Science,* **333**(6047), 1244–8.
6. Descartes, R. (1664) The treatise on man, Clerselier, .
7. Kogge, W. and Richter, M. (2013) Synthetic biology and its alternatives. Descartes, Kant and the idea of engineering biological machines. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences,* **44**(2), 181–189.
8. Szostak, J. W., Bartel, D. P., and Luisi, P. L. (2001) Synthesizing life. *Nature,* **409**(6818), 387–90.
9. Saha, R. and Chen, I. A. (2015) Origin of Life: Protocells Red in Tooth and Claw. *Current Biology,* **25**(24), R1175–R1177.
10. Kant, I., Guyer, P., and Matthews, E. (2000) Critique of the Power of Judgment, Cambridge University Press, .
11. Solé, R. V., Munteanu, A., Rodriguez-Caso, C., and Macía, J. (2007) Synthetic protocell biology: from reproduction to computation. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences,* **362**(1486), 1727–1739.
12. Pratt, V. and Brook, I. (1996) Goethe's archetype and the Romantic concept of the self. *Studies in History and Philosophy of Science Part A,* **27**(3), 351–365.
13. Peret, J. and Catal, J. (2007) The renaissance of synthetic biology. *Biological Theory,* **2**, 128–130.
14. Monod, J. and Franoise, J. (1961) Teleonomic mechanisms in cellular metabolism, growth, and differentiation.. *Cold Spring Harb Symp Quant Biol,* **26**, 389–401.
15. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) Molecular Biology of the Cell, Garland Science, 4 edition.
16. Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, Number v. 1 in Molecular Cloning: A Laboratory ManualCold Spring Harbor Laboratory Press, .
17. Hobom, B. (1980) Surgery of genes. At the doorstep of synthetic biology. *Medizin. Klinik,* **75**, 14–21.
18. McGinn, S. and Gut, I. G. (2013) DNA sequencing spanning the generations. *New Biotechnology,* **30**(4), 366–372.
19. Perrimon, N. and Mathey-Prevot, B. (2007) Applications of high-throughput RNA interference screens to problems in cell and developmental biology. *Genetics,* **175**(1), 7–16.
20. Braun, P. and LaBaer, J. (2003) High throughput protein production for functional proteomics. *Trends in biotechnology,* **21**(9), 383–8.
21. Welti, R. and Wang, X. (2004) Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. *Current Opinion in Plant Biology,* **7**(3), 337–344.
22. Kitano, H. (2001) Foundations of Systems Biology, The MIT Press, .
23. Westerhoff, H. V. and Palsson, B. O. (2004) The evolution of molecular biology into systems biology. *Nature Biotech,* **22**, 1249–1252.
24. Bruggeman, F. J. and Westerhoff, H. V. (2007) The nature of systems biology. *Trends in Microbiology,* **15**(1), 45–50.
25. Hartwell, L., Hopfield, J., Leibler, S., and Murray, A. (1999) From molecular to modular cell biology. *Nature,* **402**, C47–C52.
26. Rawls, R. (2000) Synthetic Biology' Makes Its Debut. *Chem. Eng. News,* **78**, 49–53.
27. Benner, S. a. and Sismour, a. M. (2005) Synthetic biology. *Nature reviews. Genetics,* **6**(7), 533–43.
28. Luisi, P. L. (2007) Chemical aspects of synthetic biology. *Chemistry & biodiversity,* **4**(4), 603–21.
29. Chiarabelli, C., Stano, P., and Luisi, P. L. (2009) Chemical approaches to synthetic biology. *Current Opinion in Biotechnology,* **20**(4), 492–497.
30. Hutchison, C. A. et al. (1999) Global transposon mutagenesis and a minimal Mycoplasma genome. *Science,* **286**(5447), 2165–2169.
31. Chiarabelli, C., Stano, P., and Luisi, P. L. (2013) Chemical synthetic biology: a mini-review. *Frontiers in Microbiology,* **4**(September), 1–7.
32. Hutchison, C. A., Chuang, R.-Y., Noskov, V. N., Assad-Garcia, N., Deerinck, T. J., et al. (2016) Design and synthesis of a minimal bacterial genome. *Science,* **351**(6280), aad6253.
33. Vidal, M. (2009) A unifying view of 21st century systems biology. *FEBS Letters,* **583**(24), 3891–3894.
34. Cameron, D. E., Bashor, C. J., and Collins, J. J. (2014) A brief history of synthetic biology. *Nature reviews. Microbiology,* **12**(5), 381–90.
35. Ausländer, S. and Fussenegger, M. (2013) From gene switches to mammalian designer cells: present and future prospects. *Trends in Biotechnology,* **31**(3), 155–168.
36. Isaacs, F. J., Dwyer, D. J., and Collins, J. J. (2006) RNA synthetic biology. *Nature biotechnology,* **24**(5), 545–554.
37. Grunberg, R. and Serrano, L. (2010) Strategies for protein synthetic biology. *Nucleic Acids Research,* **38**(8), 2663–2675.
38. McAdams, H. H. and Arkin, A. (2000) Gene regulation: Towards a circuit engineering discipline. *Current Biology,* **10**(8), 318–320.
39. Becskei, a. and Serrano, L. (2000) Engineering stability in gene networks by autoregulation. *Nature,* **405**(6786), 590–593.
40. Tabor, J. J., Salis, H. M., Simpson, Z. B., Chevalier, A. a., Levskaya, A., Marcotte, E. M., Voigt, C. a., and Ellington, A. D. (2009) A synthetic genetic edge detection program. *Cell,* **137**(7), 1272–81.
41. Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000) Construction of a genetic toggle switch in Escherichia coli.. *Nature,* **403**(6767), 339–42.
42. Elowitz, M. B. and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature,* **403**(6767), 335–8.
43. Weiss, R. and Basu, S. (2002) The Device Physics of Cellular Logic Gates. *First Workshop on Non-Silicon Computation,* pp. 54–61.
44. Weiss, R. and Knight, T. (2000) Lecture Notes in Computer Science, Vol. 7246, Springer-Verlag, .
45. Weiss, R., Basu, S., Hooshangi, S., Kalmbach, A., and Karig, D. (2003) Genetic Circuit Building Blocks for Cellular Computation , Communications , and Signal Processing. *Natural Computing,* pp. 1–40.
46. Garcia-Ojalvo, J., Elowitz, M. B., and Strogatz, S. H. (2004) Modeling a synthetic multicellular clock: repressilators coupled by quorum sensing. *Proceedings of the National Academy of Sciences of the United States of America,* **101**(30), 10955–60.
47. Hooshangi, S. and Bentley, W. E. (2008) From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. *Current opinion in biotechnology,* **19**(6), 550–5.
48. Carbonell-Ballestero, M., Duran-Nebreda, S., Montañez, R., Solé, R., Macía, J., and Rodríguez-Caso, C. (2014) A bottom-up characterization of transfer functions for synthetic biology designs: lessons from enzymology.. *Nucleic acids research,* **42**(22), 14060–14069.
49. Garg, N., Manchanda, G., and Kumar, A. (2014) Bacterial quorum sensing: circuits and applications. *Antonie van Leeuwenhoek,* **105**(2), 289–305.
50. Hennig, S., Rödel, G., and Ostermann, K. (2015) Artificial cell-cell communication as an emerging tool in synthetic biology applications. *Journal of Biological Engineering,* **9**(1), 13.
51. Davis, R. M., Muller, R. Y., and Haynes, K. A. (2015) Can the natural diversity of quorum-sensing advance synthetic biology?. *Frontiers in bioengineering and biotechnology,* **3**.
52. Maharbiz, M. M. (2012) Synthetic multicellularity. *Trends in Cell*

Biology, **22**(12), 617–623.
53. You, L., Cox, R. S., Weiss, R., and Arnold, F. H. (2004) Programmed population control by cell–cell communication and regulated killing. *Nature,* **428**(6985), 868–871.
54. Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., and Weiss, R. (2005) A synthetic multicellular system for programmed pattern formation. *Nature,* **434**(7037), 1130–4.
55. Martin, V. J. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003) Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. *Nature biotechnology,* **21**(7), 796–802.
56. Stephanopoulos, G. (2012) Synthetic biology and metabolic engineering. *ACS synthetic biology,* **1**(11), 514–525.
57. Becskei, A., Séraphin, B., and Serrano, L. (2001) Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *The EMBO journal,* **20**(10), 2528–35.
58. Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature,* **440**(7086), 940–943.
59. Paddon, C. J. et al. (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature,* **496**(7446), 528–532.
60. Ajo-Franklin, C. M., Drubin, D. a., Eskin, J. a., Gee, E. P. S., Landgraf, D., Phillips, I., and Silver, P. a. (2007) Rational design of memory in eukaryotic cells. *Genes & development,* **21**(18), 2271–6.
61. Burrill, D. R. and Silver, P. a. (2010) Making Cellular Memories. *Cell,* **140**(1), 13–18.
62. Kramer, B. P. et al. (2004) An engineered epigenetic transgene switch in mammalian cells. *Nature Biotechnology,* **22**(7), 867–870.
63. Weber, W. and Fussenegger, M. (2002) Artificial mammalian gene regulation networks-novel approaches for gene therapy and bioengineering. *Journal of biotechnology,* **98**(2-3), 161–87.
64. Kramer, B. P., Fischer, C., and Fussenegger, M. (2004) BioLogic gates enable logical transcription control in mammalian cells. *Biotechnol Bioeng,* **87**(4), 478–484.
65. Hasty, J., McMillen, D., and Collins, J. J. (2002) Engineered gene circuits. *Nature,* **420**(6912), 224–30.
66. Kærn, M., Blake, W. J., and Collins, J. (2003) The Engineering of Gene Regulatory Networks. *Annual Review of Biomedical Engineering,* **5**(1), 179–206.
67. Tamsir, A., Tabor, J. J., and Voigt, C. A. (2011) Robust multicellular computing using genetically encoded NOR gates and chemical wires. *Nature,* **469**(7329), 212–215.
68. Moon, T. S., Lou, C., Tamsir, A., Stanton, B. C., and Voigt, C. A. (2012) Genetic programs constructed from layered logic gates in single cells. *Nature,* **491**(7423), 249–253.
69. Regot, S. et al. (2011) Distributed biological computation with multicellular engineered networks. *Nature,* **469**(7329), 207–11.
70. Macía, J., Posas, F., and Solé, R. V. (2012) Distributed computation: the new wave of synthetic biology devices. *Trends in Biotechnology,* **30**(6), 342–349.
71. Macia, J. and Sole, R. (2014) How to Make a Synthetic Multicellular Computer. *PLoS ONE,* **9**(2), e81248.
72. Groth, A. C. and Calos, M. P. (2004) Phage Integrases: Biology and Applications. *Journal of Molecular Biology,* **335**(3), 667–678.
73. Fogg, P. C., Colloms, S., Rosser, S., Stark, M., and Smith, M. C. (2014) New Applications for Phage Integrases. *Journal of Molecular Biology,* **426**(15), 2703–2716.
74. Siuti, P., Yazbek, J., and Lu, T. K. (2013) Synthetic circuits integrating logic and memory in living cells. *Nature biotechnology,* **31**(5), 448–52.
75. Purcell, O. and Lu, T. K. (2014) Synthetic analog and digital circuits for cellular computation and memory. *Current Opinion in Biotechnology,* **29**, 146–155.
76. Bonnet, J., Yin, P., Ortiz, M. E., Subsoontorn, P., and Endy, D. (2013) Amplifying genetic logic gates. *Science,* **340**(6132), 599–603.
77. Ausländer, S., Ausländer, D., Müller, M., Wieland, M., and Fussenegger, M. (2012) Programmable single-cell mammalian biocomputers. *Nature,* **487**(7405), 123–7.
78. Seo, S. W. et al. (2013) Synthetic biology: Tools to design microbes for the production of chemicals and fuels. *Biotechnology Advances,* **31**(6), 811–817.
79. Kis, Z., Sant'Ana Pereira, H., Homma, T., Pedrigi, R. M., and Krams, R. (2015) Mammalian synthetic biology: emerging medical applications. *Journal of the Royal Society, Interface,* **12**, 1–18.
80. Chappell, J. et al. (2013) The centrality of RNA for engineering gene expression. *Biotechnology Journal,* **8**(12), 1379–1395.
81. Qi, L. S. and Arkin, A. P. (2014) A versatile framework for microbial engineering using synthetic non-coding RNAs. *Nature Reviews Microbiology,* **12**(5), 341–354.
82. Isaacs, F. J. et al. (2004) Engineered riboregulators enable post-transcriptional control of gene expression. *Nature Biotechnology,* **22**(7), 841–847.
83. Bayer, T. S. and Smolke, C. D. (2005) Programmable ligand-controlled riboregulators of eukaryotic gene expression. *Nature biotechnology,* **23**(3), 337–43.
84. Bradley, R. W., Buck, M., and Wang, B. (2015) Tools and principles for microbial gene circuit engineering. *Journal of Molecular Biology,*.
85. Benenson, Y. (2012) Synthetic biology with RNA: progress report. *Current opinion in chemical biology,* **16**(3-4), 278–84.
86. Henrichs, T. et al. (2005) Target-directed proteolysis at the ribosome. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(12), 4246–51.
87. Grünberg, R., Leckner, J., and Nilges, M. (2004) Complementarity of Structure Ensembles in Protein-Protein Binding. *Structure,* **12**(12), 2125–2136.
88. Kent, S. (2004) Novel forms of chemical protein diversity – in nature and in the laboratory. *Current opinion in biotechnology,* **15**(6), 607–14.
89. Grünberg, R., Nilges, M., and Leckner, J. (2006) Flexibility and Conformational Entropy in Protein-Protein Binding. *Structure,* **14**(4), 683–693.
90. Boyle, P. M. and Silver, P. a. (2009) Harnessing nature's toolbox: regulatory elements for synthetic biology. *Journal of the Royal Society, Interface / the Royal Society,* **6 Suppl 4**(March), S535–46.
91. Park, S.-h., Zarrinpar, A., and Lim, W. A. (2003) Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science,* **299**(5609), 1061–4.
92. Bashor, C. J., Helman, N. C., Yan, S., and Lim, W. A. (2008) Pathway Signaling Dynamics. *Science,* **319**(March), 1539–1543.
93. Whitaker, W. R., Davis, S. a., Arkin, a. P., and Dueber, J. E. (2012) Engineering robust control of two-component system phosphotransfer using modular scaffolds. *Proceedings of the National Academy of Sciences,* **109**(44), 18090–18095.
94. Dueber, J. E. et al. (2009) Synthetic protein scaffolds provide modular control over metabolic flux. *Nature Biotechnology,* **27**(8), 753–759.
95. Chau, A. H., Walter, J. M., Gerardin, J., Tang, C., and Lim, W. a. (2012) Designing synthetic regulatory networks capable of self-organizing cell polarization. *Cell,* **151**(2), 320–332.
96. Olson, E. J. and Tabor, J. J. (2012) Post-translational tools expand the scope of synthetic biology. *Current Opinion in Chemical Biology,* **16**(3-4), 300–306.
97. Khalil, A. and Collins, J. (2010) Synthetic biology: applications come of age. *Nature Reviews Genetics,* **11**(5), 367–379.
98. Ruder, W. C., Lu, T., and Collins, J. J. (2011) Synthetic biology moving into the clinic. *Science,* **333**(6047), 1248–52.
99. Weber, W. and Fussenegger, M. (2012) Emerging biomedical applications of synthetic biology. *Nature Reviews Genetics,* **13**(1), 21–35.
100. Ferrari, S. et al. (2007) Mutations of the Igβ gene cause agammaglobulinemia in man. *The Journal of Experimental Medicine,* **204**(9), 2047–2051.
101. Becker, M. M. et al. (2008) Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc Natl Acad Sci U S A,* **105**(50), 19944–19949.
102. Dwyer, D. J., Kohanski, M. A., Hayete, B., and Collins, J. J. (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli. *Molecular Systems Biology,* **3**(91).
103. Weber, W. et al. (2008) A synthetic mammalian gene circuit reveals antituberculosis compounds. *Proceedings of the National Academy of Sciences of the United States of America,* **105**(29), 9994–9998.
104. Amidi, M., de Raad, M., Crommelin, D. J. a., Hennink, W. E., and Mastrobattista, E. (2011) Antigen-expressing immunostimulatory liposomes as a genetically programmable synthetic vaccine. *Systems and Synthetic Biology,* **5**(1), 21–31.
105. Duan, F., Curtis, K. L., and March, J. C. (2008) Secretion of insulinotropic proteins by commensal bacteria: Rewiring the gut to treat diabetes. *Applied and Environmental Microbiology,* **74**(23), 7437–7438.
106. Rao, S. et al. (2005) Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide.

Proceedings of the National Academy of Sciences of the United States of America, **102**(34), 11993–11998.
107. Farrar, M. D. et al. (2005) Engineering of the gut commensal bacterium Bacteroides ovatus to produce and secrete biologically active murine interleukin-2 in response to xylan. *Journal of applied microbiology,* **98**(5), 1191–7.
108. Lu, T. K. and Collins, J. J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America,* **104**(27), 11197–202.
109. Lu, T. K. and Collins, J. J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(12), 4629–34.
110. Wu, C.-Y., Rupp, L. J., Roybal, K. T., and Lim, W. a. (2015) Synthetic biology approaches to engineer T cells. *Current Opinion in Immunology,* **35**, 123–130.
111. Kemmer, C., Gitzinger, M., Daoud-El Baba, M., Djonov, V., Stelling, J., and Fussenegger, M. (2010) Self-sufficient control of urate homeostasis in mice by a synthetic circuit. *Nature Biotechnology,* **28**(4), 355–360.
112. Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., and Keasling, J. D. (2008) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Current Opinion in Biotechnology,* **19**(6), 556–563.
113. Clomburg, J. M. and Gonzalez, R. (2010) Biofuel production in Escherichia coli: The role of metabolic engineering and synthetic biology. *Applied Microbiology and Biotechnology,* **86**(2), 419–434.
114. Jarboe, L. R., Zhang, X., Wang, X., Moore, J. C., Shanmugam, K. T., and Ingram, L. O. (2010) Metabolic engineering for production of biorenewable fuels and chemicals: Contributions of synthetic biology. *Journal of Biomedicine and Biotechnology,* **2010**.
115. Colin, V. L., Rodríguez, A., and Cristbal, H. A. (2011) The role of synthetic biology in the design of microbial cell factories for biofuel production. *Journal of Biomedicine and Biotechnology,* **2011**.
116. Alper, H. and Stephanopoulos, G. (2009) Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential?. *Nature reviews. Microbiology,* **7**(10), 715–723.
117. Steen, E. J. et al. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature,* **463**(7280), 559–562.
118. Atsumi, S., Hanai, T., and Liao, J. C. (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature,* **451**(7174), 86–89.
119. Huo, Y.-X. et al. (2011) Conversion of proteins into biofuels by engineering nitrogen flux. *Nature Biotechnology,* **29**(4), 346–351.
120. Yim, H. et al. (2011) Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nature Chemical Biology,* **7**(7), 445–452.
121. Choi, Y. J. and Lee, S. Y. (2013) Microbial production of short-chain alkanes. *Nature,* **502**(7472), 571–574.
122. Anesiadis, N., Cluett, W. R., and Mahadevan, R. (2008) Dynamic metabolic engineering for increasing bioprocess productivity. *Metabolic Engineering,* **10**(5), 255–266.
123. Holtz, W. J. and Keasling, J. D. (2010) Engineering Static and Dynamic Control of Synthetic Pathways. *Cell,* **140**(1), 19–23.
124. Zhang, F., Carothers, J. M., and Keasling, J. D. (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nature Biotechnology,* **30**(4), 354–359.
125. Potthast, T. (2009) Paradigm shifts versus fashion shifts?. *EMBO reports,* **10**(Special Issue), 42–45.
126. Soyer, O. S. and Swain, P. S. (2013) Systems and synthetic biology underpinning biotechnology. *Current Opinion in Biotechnology,* **24**(4), 735–736.
127. Becker, A. (2014) Synthetic biology changing the face of biotechnology. *Journal of Biotechnology,* **169**(1), 3.
128. Brent, R. (2004) A partnership between biology and engineering. *Nature biotechnology,* **22**(10), 1211–4.
129. Purnick, P. E. M. and Weiss, R. (2009) The second wave of synthetic biology: from modules to systems. *Nature reviews. Molecular cell biology,* **10**(6), 410–22.
130. Elowitz, M. and Lim, W. a. (2010) Build life to understand it.. *Nature,* **468**(7326), 889–890.
131. Serrano, L. (2007) Synthetic biology: promises and challenges. *Molecular systems biology,* **3**(158), 158.
132. Slusarczyk, A. L., Lin, A., and Weiss, R. (2012) Foundations for the design and implementation of synthetic genetic circuits. *Nature reviews. Genetics,* **13**(6), 406–20.
133. Gardner, T. S. and Hawkins, K. (2013) Synthetic Biology: evolution or revolution? A co-founder's perspective. *Current Opinion in Chemical Biology,* **17**(6), 871–877.
134. Gardner, T. S. (2013) Synthetic biology: from hype to impact. *Trends in Biotechnology,* **31**(3), 123–125.
135. Ball, P. (2004) Synthetic Biology: Starting from scratch. *Nature,* **431**, 624–626.
136. Cheng, A. A. and Lu, T. K. (2012) Synthetic biology: an emerging engineering discipline. *Annual review of biomedical engineering,* **14**, 155–178.
137. Endy, D. (2005) Foundations for engineering biology. *Nature,* **438**(7067), 449–53.
138. Andrianantoandro, E., Basu, S., Karig, D. K., and Weiss, R. (2006) Synthetic biology: new engineering rules for an emerging discipline. *Molecular systems biology,* **2**, 2006.0028.
139. Heinemann, M. and Panke, S. (2006) Synthetic biology–putting engineering into biology. *Bioinformatics (Oxford, England),* **22**(22), 2790–9.
140. Russell, a. L. (2005) Standardization in History : A Review Essay with an Eye to the Future. In *The Standards Edge: Future Generations* Sherrie Bolin.
141. International Telecommunication Union (2014) Understanding patents, competition & standardization in an interconnected world, ITU, .
142. Noble, D. (2013) AMERICA BY DESIGN, Knopf Doubleday Publishing Group, .
143. Wikipedia Standardization. (2016) [Online; accessed 02-February-2016].
144. Ellis, T., Adie, T., and Baldwin, G. S. (2011) DNA assembly for synthetic biology: from parts to pathways and beyond. *Integrative biology : quantitative biosciences from nano to macro,* **3**(2), 109–118.
145. Canton, B., Labno, A., and Endy, D. (2008) Refinement and standardization of synthetic biological parts and devices. *Nature biotechnology,* **26**(7), 787–93.
146. Arkin, A. (2008) Setting the standard in synthetic biology. *Nature biotechnology,* **26**(7), 771–774.
147. Instruments, T. (2003) Digital Logic Pocket Data Book, Texas Instruments, .
148. iGEM MIT Registry of Standard Biological Parts. (2004) 2016-02-09.
149. Knight, T. (2003) Idempotent Vector Design for Standard Assembly of Biobricks. *MIT Artificial Intelligence Laboratory,* pp. 1–11.
150. Shetty, R. P., Endy, D., and Knight, T. F. (2008) Engineering BioBrick vectors from BioBrick parts. *Journal of biological engineering,* **2**, 5.
151. Peccoud, J. et al. (2008) Targeted development of registries of biological parts. *PloS one,* **3**(7), e2671.
152. Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods,* **6**(5), 343–345.
153. Engler, C., Kandzia, R., and Marillonnet, S. (2008) A One Pot, One Step, Precision Cloning Method with High Throughput Capability. *PLoS ONE,* **3**(11), e3647.
154. Kosuri, S. and Church, G. M. (2014) Large-scale de novo DNA synthesis: technologies and applications. *Nature Methods,* **11**(5), 499–507.
155. Frank, S. a. (2013) Input-output relations in biological systems: measurement, information and the Hill equation. *Biology direct,* **8**(1), 31.
156. Ang, J., Harris, E., Hussey, B. J., Kil, R., and McMillen, D. R. (2013) Tuning response curves for synthetic biology. *ACS Synthetic Biology,* **2**(10), 547–567.
157. Cox, R. S., Surette, M. G., and Elowitz, M. B. (2007) Programming gene expression with combinatorial promoters. *Molecular Systems Biology,* **3**(145), 145.
158. Chappell, J., Jensen, K., and Freemont, P. S. (2013) Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Research,* **41**(5), 3471–3481.
159. Kosuri, S. et al. (2013) Composability of regulatory sequences controlling transcription and translation in Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America,* **110**(34), 14024–9.
160. BIOFAB International Open Facility Advancing Biotechnology. (2011) 2016-02-09.
161. Mutalik, V. K. et al. (2013) Quantitative estimation of activity and quality for collections of functional genetic elements. *Nature Methods,*

10(4), 347–353.

162. Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q.-A., Tran, A. B., Paull, M., Keasling, J. D., Arkin, A. P., and Endy, D. (mar, 2013) Precise and reliable gene expression via standard transcription and translation initiation elements.. *Nature methods,* **10**(4), 354–360.
163. Technopedia Abstraction. (2016) [Online; accessed 02-February-2016].
164. Webopedia Abstraction. (2016) [Online; accessed 02-February-2016].
165. Mukherji, S. and van Oudenaarden, A. (2009) Synthetic biology: understanding biological design from synthetic circuits. *Nature reviews. Genetics,* **10**(12), 859–71.
166. Bashor, C. J., Horwitz, A. A., Peisajovich, S. G., and Lim, W. A. (2010) Rewiring Cells: Synthetic biology as a tool to interrogate the organizational principles of living systems. *Annual review of biophysics,* pp. 515–537.
167. Del Vecchio, D., Ninfa, A. J., and Sontag, E. D. (2008) Modular cell biology: retroactivity and insulation. *Molecular Systems Biology,* **4**(161).
168. Endy, D. and Brent, R. (2001) Modelling cellular behaviour. *Nature,* **409**(6818), 391–395.
169. Chen, Y. Y., Galloway, K. E., and Smolke, C. D. (2012) Synthetic biology: advancing biological frontiers by building synthetic systems. *Genome Biology,* **13**(2), 240.
170. Church, G. M., Elowitz, M. B., Smolke, C. D., Voigt, C. a., and Weiss, R. (2014) Realizing the potential of synthetic biology. *Nature reviews. Molecular cell biology,* **15**(4), 289–94.
171. Whitford, D. (2005) Proteins. Structure and Function., John Wiley and Sons, .
172. Liberles, D. A. et al. (2012) The interface of protein structure, protein biophysics, and molecular evolution. *Protein Science,* **21**(6), 769–785.
173. Venter, J. C. et al. (2001) The Sequence of the Human Genome. *Science,* **291**(February), 1304–1351.
174. Maher, B. (2012) ENCODE: The human encyclopaedia. *Nature,* **489**(7414), 46–48.
175. Qu, H. and Fang, X. (2013) A Brief Review on the Human Encyclopedia of DNA Elements (ENCODE) Project. *Genomics, Proteomics and Bioinformatics,* **11**(3), 135–141.
176. Kwok, R. (2010) Five hard truths for synthetic biology. *Nature,* **463**(January).
177. Cardinale, S. and Arkin, A. P. (2012) Contextualizing context for synthetic biology - identifying causes of failure of synthetic biological systems. *Biotechnology Journal,* **7**(7), 856–866.
178. Leonard, E., Nielsen, D., Solomon, K., and Prather, K. J. (2008) Engineering microbes with synthetic biology frameworks. *Trends in biotechnology,* **26**(12), 674–81.
179. Glick, B. R. (1995) Metabolic load and heterologous gene expression. *Biotechnology Advances,* **13**(2), 247–261.
180. Klumpp, S., Zhang, Z., and Hwa, T. (2009) Growth rate-dependent global effects on gene expression in bacteria. *Cell,* **139**(7), 1366–75.
181. Scott, M., Mateescu, E. M., Zhang, Z., and Hwa, T. (2010) Interdependence of Cell Growth Origins and Consequences. *Science,* **330**(November), 1099–1102.
182. Gyorgy, A., Jiménez, J. I., Yazbek, J., Huang, H.-H., Chung, H., Weiss, R., and Del Vecchio, D. (2015) Isocost Lines Describe the Cellular Economy of Genetic Circuits. *Biophysical Journal,* **109**(3), 639–646.
183. Lynch, S. A. and Gill, R. T. (2012) Synthetic biology: New strategies for directing design. *Metabolic Engineering,* **14**(3), 205–211.
184. Larson, M. H., Gilbert, L. a., Wang, X., Lim, W. a., Weissman, J. S., and Qi, L. S. (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nature protocols,* **8**(11), 2180–96.
185. Doudna, J. A. and Charpentier, E. (2014) The new frontier of genome engineering with CRISPR-Cas9. *Science,* **346**(6213), 1258096–1258096.
186. Sander, J. D. and Joung, J. K. (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology,* **32**(4), 347–55.
187. Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L. A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology,* **31**(3), 233–239.
188. DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., and Church, G. M. (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. *Nucleic acids research,* **41**(7), 4336–43.
189. Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., and Doudna, J. (2013) RNA-programmed genome editing in human cells. *eLife,* **2013**(2), 1–9.

4.2 On Genetic Device's Characterization

Exploring how to improve standardization via better characterization of the synthetic devices

As stressed in the introduction, *standardization* is one of the principles that was borrowed from other engineering disciplines and applied to biology to try to turn Synthetic Biology into an engineering discipline. A proper standardization is *conditio sine qua non* for the success of any engineering discipline, necessary but not enough. As pointed out by other authors, standardization has to be applied to methods, processes and other relevant issues of the field, for instance, to part's definition and measurement and to system's operating conditions [Endy, 2005, Canton et al., 2008, Slusarczyk et al., 2012, Arkin, 2013].

Ten years after the idea of applying these principles popped up, several initiatives have been assayed towards the development and consolidation of these principles. The '*RSBP*' held at the MIT [iGEM, 2004], the *BioBrick* format for the assembly of 'parts' created by T.Knight [Knight, 2003] and the *Datasheet* proposed by Canton [Canton et al., 2008] are, among others, examples of these efforts regarding the establishment and consolidation of standardization as a fundamental principle in the field.

Within standardization, *characterization* is an essential step in enabling the construction of more complicated devices and systems based on the previously characterized parts or building blocks. It should provide an accurate description or representation of such parts, which, ideally, should include all the necessary information for a correct combination of these parts to create the desired devices or systems. As an example, the characterization of genetic regulable devices often includes, among other relevant characteristics common to other kind of devices, a (quantitative) input-output representation. For instance, the one that relates a given regulator (i.e. input, such as an activator or inhibitor signal) and the device's response to it (i.e. output, such as a fluorescent protein). This relation be-

tween the input and the output of a given device is called transfer function, a concept inspired by electronics.

The idea behind repositories and other platforms of the kind for Synthetic Biology is that they should promote the creation of complex devices without the need of starting every project from zero. These repositories physically store the genetic parts and devices and virtually gather and share all relevant information regarding them -usually collected in reports or datasheets. Canton and colleagues provided a nice example of a Datasheet comprising a lot of information of a regulable device, including its transfer function and information of its dynamic performance [Canton et al., 2008]. However, this case is more an exception than a rule within the community. For instance, many of the parts and devices included in an important registry such as the *RSBP* lack proper characterization and its correspondent datasheets [Gardner and Hawkins, 2013].

Nevertheless, such lack of parts characterization is not the only problem that hinders the use of such registries. The truth is that, even though some parts may be characterized, when these are used and combined to create more complex devices the result is often not as expected. Designs and predictions based on its previous characterizations fail too frequently. It seems obvious that the problem not only resides in the lack of characterization but also in how this is performed.

As in electric engineering, response curves of genetic devices often display non-linear behavior. A widely used mathematical tool for describing and modeling such kind of behaviors are Hill functions, mainly due to their simplicity and versatility in fitting experimental data. Hence, when characterizing regulable devices this is the tool *par excellence*. However, Hill functions are just empirical approximations, i.e fittings of experimental data. In this section, the article presented reveals the weak point of the use of Hill functions since they have shown unable to transcend the particular set of experimental conditions in which the data is obtained. Although they give information about the system's affinity and cooperativity, provide a limited predictive value due to the frequent disconnection between the mathematical formalism and the real biological mechanism that tries to describe. Thus, better frameworks for the characterization

of genetic regulable devices should be considered in order to improve the outcome of synthetic biology designs made from previously characterized parts of this kind.

Consequently, we propose to develop descriptive models with predictive power based on the underlying biological mechanisms at play en each case. In this scenario, and considering our case-of-study-, the enzymological formalism based on Michaelis-Menten kinetics has revealed as a really useful one. This approach provides causal connections between the experimental data of the transfer function and the underlying responsible biological mechanisms. As a case-of-study, we use a well-known regulable genetic device in the field, a lactone-inducible system, to experimentally validate this approach.

The article that is attached below deals with the characterization of the behavior of a synthetic device that is designed to sense molecules based on a Lux receptor from the *QS* (signal) system. Our results show how thanks to this novel framework we confer to transfer functions a better predictive power. Using this approach we were able to quantitatively predict the effect in the affinity response and amplitude signal of the abovementioned synthetic sensor when we varied the levels of receptor. In this sense, our results question the suitability of the widely used approach based on Hill functions, which has less predictive value and might suggest cooperativity mechanisms when indeed these are neither needed nor demonstrated.

Carbonell-Ballestero M, Duran-Nebreda S, Montañez R, Solé R, Macía J, Rodríguez-Caso C. A bottom-up characterization of transfer functions for synthetic biology designs: lessons from enzymology. Nucleic Acids Res. 2014 Dec 16;42(22):14060-9. doi: 10.1093/nar/gku964.

4.3 On Genetic Load

Exploring circuit-host interactions and its relation with the lack of proper modularity and the metabolic load.

As we have exposed in the introduction chapter, modularity is a property that is often taken for granted when designing synthetic biology devices and systems. If this was true, after creating a number of devices and thoroughly characterizing them, one should be able to combine them in a predictable way to create a vast number of more complex devices and systems. However, as we have also discussed, a number of different factors stand in the way of synthetic biologists [Kwok, 2010].

Many of these factors are related to diverse contextual issues, for instance, the nature of the host (e.g. its genetic background) or the environment (e.g. the media in which the host grows) [Cardinale and Arkin, 2012]. These issues acquire often more relevance when designs grow in size and complexity, situations in which incapacity of the cell to host the circuit and make it work are referred to as a *metabolic load* problem that, besides, affects cells normal physiology.

Exploring in deeper detail this situation and the evidences provided by other authors, it seems clear there has to be a dependence between the genetic parts, modules or whole circuits and the hosts that provides them with the environment and resources they need to properly perform their function. However, to what extent these dependencies influence and condition the performance of the different modules or circuits is not clear. Given the fact that design's failures tend to appear when large circuits are used, a limited amount of cell resources and machinery seem to be a possible explanation or scenario [Peretti and Bailey, 1987, Glick, 1995, Scott et al., 2010].

The situation of a number of coexisting demands (competing for) and a limited amount of resources is not unique of biology. Reinforcing the communicating channels between electrical engineering and synthetic bi-

ology, we found a similar situation, for instance, when we have a real power supply and several electric resistances connected to it. In electric circuits, the well-known Ohm's law describes the behavior of such systems only in terms of a few key elements like voltage, intensity and resistance [Nilsson and A, 2011]. For instance, given a power supply and a set of resistances connected to it in a series, this law describes the relationship between the electric energy consumed in each electric load, with respect to the others. Likewise, drawing an analogy between electric theory and 'genetic theory', *can we think of cells or hosts as power supplies and of genetic loads as resistances connected in a series to this power source?*

Considering all that has been exposed, in our second research article we explore a general framework that demonstrates how the competition for the limited amount of resources within a cell conditions the expression of the different genetic demands (i.e. genetic loads) that coexist in it. This, in turn, establishes an interdependence between the different genetic loads within a cell similar to what we see in electric theory. This unexpected interdependences act against the assumed modularity of the system and orthogonality between the different modules or genetic loads that integrate such modules.

Thus, our work aims to shed light on the role of genetic load on circuit's performance. Using bacteria as our model organism, we theoretically and experimentally demonstrate that gene expression is conditioned by genetic load due to the competition for the resources that arises from a limited amount of resources for a given set of genetic loads. Our framework provides tools for the predictive treatment of this load and highlights the similarities that emerge between different systems where there is competition for limited resources and regulation activities, that go beyond electricity.

Carbonell-Ballestero M, Garcia-Ramallo E, Montañez R, Rodriguez-Caso C, Macía J. Dealing with the genetic load in bacterial synthetic biology circuits: convergences with the Ohm's law. Nucleic Acids Res. 2016 Jan 8;44(1):496-507. doi: 10.1093/nar/gkv1280

Chapter 5

DISCUSSION

When it comes to scientific discussion, there's no authority that is beyond reason or above arguments, facts and evidences[1].

[1]This is a sentence that proceeds from an oral conversation -not literally reproduced- with my Thesis co-director, *Carlos Rodríguez-Caso*. Within my first days in the lab, while he was explaining me that in terms of group management he was the authority, he emphasized that when it comes to scientific discussion there is no authority at all.

In this **Discussion** chapter, the reader will find four sections. The first three sections, following the structure that has been employed throughout this Thesis, correspond to each of the three objectives that were set and to its subsequent results sections. Finally, I add a fourth section in which I provide brief reflections, from a general perspective, of my Thesis, connecting with the general objective that was also formulated at the beginning of the **Objectives** chapter.

5.1 Overview of the field

The work presented here has tried to shed some light on the field of Synthetic Biology. As we have seen, this name describes many things that come together to conform this novel discipline. It has a multiplicity and variety of meanings and refers to slightly different approaches that want to make a significant difference in how technology and living systems are combined. Biology is the technology of the new century and Synthetic Biology, and more specifically its main engineering-oriented branch, aims to drives us towards this ambitious goal.

However, some people question whether there is actually any difference between what synthetic biologists do from what common biotechnologists and molecular biologists have been doing for decades, or from what systems biologists have been doing since more recently [Gardner and Hawkins, 2013]. In fact, others argue that this name, Synthetic Biology, might be just a buzzword. They argue that the coining of the name Synthetic Biology and the aim to create a discipline around it are just strategies to attract funds and enroll scientists [Kastenhofer, 2013, Bensaude Vincent, 2013]. For instance, Schyfter thinks that its estrangement from well established science would serve to demarcate the field and to assert its novelty. He thinks that its mission statement -to make biology easy to engineer- would be an (over)idealization and a construct with the purpose of directing research, shaping the nascent field and creating a community around it [Schyfter et al., 2013].

These are indeed legitimate claims and might bear part of the truth. However, other scientists agree in that the foreseeable potential uses of this discipline, which seeks to rationally and efficiently engineer living systems, are worth a try. Synthetic Biology promises to find useful solutions both to improve the human condition and the future of the planet through the development of a myriad of applications. This endeavor is indeed a great challenge and it holds some perils, however, it is no less true that its potential benefits might be even greater [Arkin and Fletcher, 2006].

Classical approaches like Biotechnology and Molecular Biology have

deeply influenced the field. In fact, they pervade many of the daily practices and methods used by synthetic biologists and are considered as precursors of this field. The key issue that should make a significant difference between these other fields and Synthetic Biology relies on concepts such as 'rational' or 'design', which point out the direction this field wants to follow. The final target is to find fundamental design principles that should allow to engineer living systems, ideally, in a systematic, straightforward and reliable way. Traditionally, this has not been the main goal of Biotechnology, in which the emphasis has been putted in finding *ad hoc* solutions for each particular situation or application [Bud, 1994].

Another discipline that has much to say about Synthetic Biology is Systems Biology. Both mean a change in the way in which biologists practice their science, combining analytic and systemic approaches to fully describe the systems under study. Furthermore, they are also merged by the use of computational tools, modeling and simulations to study *in silico* either the actual systems or the newly designed ones [Morange, 2009]. Some authors might even argue that they are indeed two faces of the same coin. They represent complementary approaches that puts us closer to *understanding* and *dealing* with living systems, being these two goals what drives Systems Biology and Synthetic Biology, respectively [Kastenhofer, 2013, Gramelsberger, 2013]. Their key difference, however, lies not only in which is the final goal they pursue, but also in their driving credo. Unlike Systems Biology, Synthetic Biology blurs the distinction between engineering and science, between making and knowing, intertwining both concepts [Keller, 2009a, Keller, 2009b]. The idea is to create a win-win interdependence: mixing theory and practice, inductive and deductive epistemological approaches, for an integrated and improved outcome [Gustafsson and Vallverdú, 2015].

An important year for Synthetic Biology was 2005, when the first two significant reviews of the field were published. While Benner and Sismour's paper in 2005 served as a classical review of what had been done in the field until that moment [Benner and Sismour, 2005], Endy's seminal review the same year acted more like a roadmap or a guideline of the steps that should be followed in order to effectively transform the field

into an engineering discipline [Endy, 2005]. He was one of the firsts scientists to propose the idea of applying engineering principles common place in other engineering disciplines, like in electric and mechanical engineering, to living systems. Following this line, the core of Synthetic Biology comes down to the applications of basic engineering principles for the straight-forward, predictable and reliable design and construction of biological systems [Smolke and Silver, 2011, Qi et al., 2015, Church et al., 2014].

Hence, the appearance of Synthetic Biology implied the encounter between engineering and biology, between the systematic and rigorous *ethos* of engineering and physics and the free-minded and typically informal culture of biological sciences [Porcar and Pereto, 2015]. Borrowing O'Malleys words, "*synthetic biology is an interesting exemplification of the tension between rational ordering and untidy making do*" [O'Malley, 2009]. Many efforts have been devoted to adapt both approaches in pursuit of a successful solution, from the standardization of genetic elements and the development of parts libraries and repositories, to the design and modeling of such systems following engineering advise or guidelines. In an ideal case, through the application of engineering principles to biology the design-build-test cycle should be accelerated and raised to the level of other engineering disciplines [Arkin, 2013, Beal, 2014]. However, as we have seen throughout this Thesis, the results so far have shown our limited success in this endeavor.

As we have intensively discussed in previous chapters, it has been widely and uncomfortably observed and documented that the behavior of engineered biological systems frequently differs from what is designed and predicted [Kwok, 2010, Cardinale and Arkin, 2012]. Different factors intervene and share a part of the responsibility of such undesired outcomes. We have already discussed the existence of several layers of uncertainty that limit the success of the engineering approaches to biological systems [Zhang et al., 2015]. Among these, the non-standard nature of biological building blocks, its context-dependence and the variability and fluctuations of living systems are important factors, not to mention our limited knowledge of such systems and of our capacity to predict

its behavior. [O'Malley, 2009, Schyfter et al., 2013, Venturelli et al., 2015, Porcar and Pereto, 2015].

All these issues represent opposing forces to the successful application of longstanding engineering principles and practices to biological systems. Although these principles have permeated the widespread and diverse synthetic biology community, its validity still remains to be proved [Way et al., 2014]. It seems that some assumptions upon which these principles are sustained, such as modularity or orthogonality, are incorrectly taken for granted [Vilanova et al., 2015]. As a consequence of all this, addressing these issues becomes of vital importance for the fruitful development of the field.

Some authors think that a comprehensive view of the failures is the strongest path towards solving this issue [Vilanova et al., 2015]. Others argue for the necessity of adapting and informing such principles to the realities of working with biological substrates [Schyfter et al., 2013]. O'Malley, for instance, states that synthetic biologists always end up designing and building in a kludge-like[2] way and that this hardly going to change. For him, the central question in Synthetic Biology is *"whether kludging can be overcome or whether it lies inseparably at the heart of both life and biological practice"* [O'Malley, 2009].

Once arrived at this point, after having discussed from a general and conceptual perspective the potentials and limitations of this field as an engineering discipline, it is time now to further explore and discuss the scientific results that are provided within this Thesis.

[2]Kludge: colloquial term for a workaround solution that is Klumsy, Lame, Ugly, Dumb, but Good Enough. It is a colloquial term used to define the achievement of a particular function regardless of the path that has led to it. It does not have to imply an elegant process or an efficient design.

5.2 Characterization of regulable devices

We have exposed the role and importance that standardization should have while developing Synthetic Biology as an engineering discipline. In electrical or mechanical engineering, for instance, the process of standardization allows to streamline design and building processes for the reliable and predictable production of a world of different applications. It is a hallmark of mature engineering fields to develop and maintain standards that should underpin mass production [Matsuoka et al., 2009, Ellis et al., 2009, Porcar and Pereto, 2015]. Moreover, standardization is closely related to a key issue that should be a mainstay of Synthetic Biology as it is in other engineering disciplines: prediction-based design.

However, as we have seen, predictability is still one of the major unconquered challenges of the field [Pasotti et al., 2012]. Living systems provide a wealth of genetic and non-genetic elements that we can define, describe and characterize as parts or building blocks. Later on, these parts should be used to design and build new devices and applications [Endy, 2005, Sprinzak and Elowitz, 2005, Arkin and Fletcher, 2006]. However, efforts devoted until nowadays to develop much needed standards and to extensively and properly characterize the parts according to such standards have proved not enough. Truly predictable design in Synthetic Biology has not yet been achieved [Slusarczyk et al., 2012, Mutalik et al., 2013a, Zhang et al., 2015].

As a consequence of this, authors such as Kosuri and colleagues have argued that instead of relying on standardization, we should rely on huge libraries where one could scan or screen for the desired behavior. In their opinion, idiosincratic interactions and context effects impede us in reaching the desirable predictability and unavoidably entail the need for the construction and testing of a large number of variants [Kosuri and Church, 2014]. Nevertheless, other authors think that the goal of designing devices and systems in a predictable way is feasible. In their opinion there is still room for improvement [Canton et al., 2008, Lucks et al., 2008, Matsuoka et al., 2009, Zhang et al., 2015].

Proposals to improve predictability in the field have been made, such

as fostering a complete checking and quantification of parts features or reinterpreting in biological terms some of the notions translated from other engineering disciplines [Arkin, 2013, Porcar and Pereto, 2015]. Furthermore, the use of tuning tools has also been suggested as well as the need to join efforts in order to develop more robust theories and formalized design processes [Arkin and Fletcher, 2006, Slusarczyk et al., 2012].

When designing, the connection of different parts to create complex devices and systems requires a correct matching between such parts, either in electronics or in Synthetic Biology. The characterization of such parts is therefore essential, as it should provide the required information to link such parts in a predictable way, including timing details, dynamic and static performance, and so on.

Most of this information is collected in datasheets. For instance, like in electrical engineering, the input-output quantitative relation (i.e. transfer function) of a given regulable device is a crucial aspect. Indeed, input-output relations are important in many biological processes, in part due to its causal relation with the underlying mechanisms [Frank, 2013]. Besides, in Synthetic Biology, they should assist the interconnection of the different parts composing the devices. To do so, we not only need to know which is the device response but also how it works and the ways in which we can tune it in a predictable way [Bintu et al., 2005, Voigt, 2006, Arpino et al., 2013].

Hence, this input-output information is used in the design process to assay different possible combinations and to predict its outcome prior to its construction and *in vivo* testing. Many of such input-output relationships display switch-like or quasi-digital sigmoidal curves, similar to those seen with cooperative enzymes and other chemical reactions [Ang et al., 2013, Frank, 2013, Ferrell and Ha, 2014, Cornish-Bowden, 2013]. The biological mechanisms responsible for such kind of responses are diverse, ranging from the classic enzymatic saturation to the different processes that might lead to ultrasensitive responses[3] (e.g.multistep cascades, multistable systems or positive feedback loops) [Cornish-Bowden,

[3]Ultrasensitivity: a property of steady-state input-output relationships that makes them switch-like in character [Ferrell and Ha, 2014].

2013, Ferrell and Ha, 2014].

The most common or systematic way to model these behaviors is by using Hill functions. They are a very flexible and effective mathematical tool that is able to fit a broad range of curve responses with only a few parameters [Goutelle et al., 2008]. Interestingly, the Hill equation is fairly similar to the classical Michaelis-Menten equation with the slight modification that results from adding an exponent (i.e. the Hill coefficient) [Goutelle et al., 2008, Cornish-Bowden, 2004]. In fact, one could say that the Michaelis-Menten approach is like a Hill approach with a Hill coefficient of one. Actually, the big increase in citations of Michaelis-Menten's original paper during the last years seems to be related to the emergence of disciplines like Systems Biology and Synthetic Biology and their broad use of Hill functions to model input-output responses [Cornish-Bowden, 2013].

However, it is important to stop here to examine the problems associated with Hill functions. The original Michaelis-Menten formulation holds a causal relation between the mathematical expression and the underlying molecular mechanisms. This is not the case of the Hill equation. Its versatility enables an easy fitting of most input-output relationships just by carefully adjusting its parameters. As a descriptive or fitting tool is really powerful, but it lacks predictive value. Its parameters do not have biological meaning or a direct mapping with any biological or physical parameter. In the end, the Hill approach becomes a semi-empirical method that is only derived from experimental observations. It is decoupled from the biological reality and beyond the precise context in which the experimental measurements are gathered it has little predictive value [Goutelle et al., 2008, Ang et al., 2013, Ferrell and Ha, 2014, Le Novère, 2015].

The assumption of cooperativity for systems fitted with Hill coefficients of two is a paradigmatic example. In principle it is thought that this number two stems for the need of dimerization inherent of a cooperative activation process. This should be the case, for example, of our system of study, the lactone-inducible-system [Alon, 2006]. However, it has been widely shown that such systems are well described by Hill equations with

coefficients around one, as in our case, and therefore claims of cooperativity based on Hill fittings with a Hill coefficient of two should be made with caution [Ramalho et al., 2016].

In the line of the results exposed here, we can say that the overuse of Hill functions in the field for making models might be negative. We should make models with more predictive power. Hill functions might be an easy and quick tool to use but they might miss important things about the underlying biological mechanisms that diminish its predictive character. Our model has been derived from the bottom-up by using classical biological knowledge and tools, like the mass action law and enzymological kinetics. This has allowed us to build a predictive model that provides insight into different aspects of our particular system.

An illustrative example of this has to do with the tuning of our system. As it has also been discussed, tunability is another key aspect that is related to engineering, and more specifically with characterization. The modification of some features of the different parts that compose a given device is aimed at facilitating its combination. This should help in correctly adapting and matching these parts according to what is designed [Sprinzak and Elowitz, 2005]. Moreover, parts tuning enables their easy reuse in different designs because it provides a certain flexibility that should facilitate the adaptation of such parts to the new situations in which they have to fit [Voigt, 2006, Slusarczyk et al., 2012].

There exist several different mechanisms for tuning the dials of synthetic biology design, and from time to time new mechanisms are discovered or new tools that are developed. A key related issue is to properly understand and relate our tuning needs regarding the model parameters with its biological implementation [Arpino et al., 2013, Ang et al., 2013]. If properly used and modeled, tuning dials may not only help us to link different parts of a given device but also to better understand the function of such parts and the devices they compose. This last aspect is key if we want to predict our device function and to foresee possible deviations from the predictions [Voigt, 2006, Arpino et al., 2013].

Interestingly, Ang and colleagues reviewed several of the options we have for tuning response curves or transfer functions in Synthetic Biol-

ogy [Ang et al., 2013]. They explore, for instance, the effects of widely used tuning mechanisms (e.g. modifying promoter or *RBS* sequences) to tune the leakyness and maximum expression levels of the curves [Gruber and Gross, 2003, Chen et al., 1994]. Beyond these well-known tuning effects, its is interesting to mention the possibility of tuning curve's affinity through the careful engineering of what they call *Signal Sensor Domains*. This refers, for example, to transcription factors able to bind exogenous external effectors (i.e. ligands), as in our case the Lux receptor and lactone molecules.

Curves affinity is a very important feature of sensor devices -like inducible systems- and the capacity to tune it through modifications of the amount of receptor is not easy to see or predict. In fact, this is illustrated in our paper. We provide an informed or grounded model with physically (and biologically) relevant parameters that can be modified, such as the strength of the *RBS*. Through this mechanism we can tune the amount of receptor of our device and anticipate how this should shift the system's affinity. After doing so, we can experimentally demonstrate the validity of our predictions.

To sump up, our Lux-inducible system serves us as a case-of-study of our approach applied to a sensor device. It is useful not only in fitting experimental data, as common Hill functions do, but also in providing predictive information regarding the effects of tuning a key part -and parameter- of the system (i.e. the RBSs) and on the role that the receptor has on the system's affinity. Moreover, it contributes to support the raising voices that question the suitability of Hill functions as one of the tools *par excellence* in the field.

This theoretical framework follows the line exposed throughout this Thesis that states the need for exploring and reinforcing the fundamental principles that must sustain the field as an engineering discipline. In this case we explore alternatives for developing better characterization frameworks while trying to improve the predictability of our designs. As future perspectives, other general scenarios and mechanisms (as, for instance, ultrasensitive responses due to multistep cascades) should be also

explored with a similar approach.

Futhermore, and just as a footnote, I would like to mention that while performing the experiments that generated the results here presented, we decided to place a *gfp* after the Lux receptor gene to check its levels and track its evolution over time and as a result of the different input concentrations. We are aware that there is not a direct mapping of gene expression between different genes within an operon, as different factors can alter the proportionality that one expects should link both genes or even new promoters may arise from such fusions, thus altering the expected output levels [Osterman et al., 2013, Levin-Karp et al., 2013, Yao et al., 2013]. However, this strategy can serve as an indicator and indeed gave us some clues that lead us to the next study. The observed but unexpected decrease in the fluorescence levels associated to the *gfp* placed in tandem with the receptor upon induction of the systems with lactone hinted to some of the issues related to the system's overloading. These issues were thoroughly studied, what later brought us to the next paper we are going to discuss now.

5.3 Genetic load and circuit-host interactions

As discussed in previous chapters, *modularity* has a central role in Synthetic Biology. In an ideal scenario, biological components would be perfectly modular and orthogonal. Devices, modules and systems created by combining such components should retain these interesting properties [Del Vecchio et al., 2008, Purnick and Weiss, 2009, Del Vecchio, 2015]. Indeed, basic building blocks such as promoters, RBSs and coding sequences are often combined with no major problems by assuming its modular nature and with the information provided by their previous characterization. The successful design and construction of simple composite-parts and devices, for instance using parts from the *RSBP*, illustrates this fact. However, it is also true that there exist some potential constrains, like those caused by the compositional context, that might compromise this modularity-based design approach [Marchisio and Stelling, 2009, Cardinale and Arkin, 2012, Gardner and Hawkins, 2013].

When designs of constructs grow in size and complexity, things get even harder. Well-characterized simple modules should be easily combined to produce new devices to perform new and more complex tasks [Endy, 2005, Kitney and Freemont, 2012]. However, in many cases, modules tend to deviate from their previously characterized behavior upon interconnection with other modules. This situation compromises the design process in Synthetic Biology and limits the success of these kinds of approaches [Liang et al., 2011, Purnick and Weiss, 2009]. Hence, lack of predictable behavior due to wrongly taking for granted modularity in the design and building processes becomes a major issue in the field [Pasotti et al., 2012, Del Vecchio, 2015].

As we have previously seen, 'the context' plays a central role at different levels in Synthetic Biology and seems to be a major cause behind the limited success rate that has characterized the field until now [Kwok, 2010, Cardinale and Arkin, 2012]. The issue of *context-dependence* is one of the major barriers for predictability in Synthetic Biology and it has been discussed by several authors [MacDonald et al., 2011, Kitney and Freemont, 2012, Gyorgy and Vecchio, 2014, Del Vecchio, 2015]. The

cellular context (e.g. hosts genetic machinery and resources), the environment (e.g. growth media, nutrients, etc) and the circuit's operating conditions (e.g. temperature, pH, etc.) are factors that strongly condition and determine the performance of synthetic circuits but that are poorly understood [Andrianantoandro et al., 2006, Smolke and Silver, 2011, Brophy and Voigt, 2014]. For instance, most of the circuits that function in one strain fail to do so in another strain, which illustrates well the dependence of synthetic circuits on the host context [Prindle and Hasty, 2012].

Hence, it is clear that host and its interactions with the circuits that are embedded in it are crucial. This seems intuitive and was in fact shown by various authors during the late 80s and the early 90s. They were participating on the expansion of DNA recombinant technology aimed at the heterologous expression of target genes via vectors within host cells. At that time, the term *metabolic load* or *metabolic burden* started to be used to describe the fact that there appeared unexpected effects on host cells, like the decrease in growth rate, due to the presence of expression vectors. Peretti and Bailey developed the first model describing hots-vector interactions through competition for resources and the effects in transcription and translation of changing the plasmid copy number, promoters and RBSs [Peretti and Bailey, 1987]. Soon after, experimental confirmations demonstrated model's predictions and an inverse relation was found between cells growth and plasmid copy number [Bentley et al., 1990, Bailey, 1993]. These results were reviewed by Glick, which also discussed some possible strategies to avoid the problems related to the metabolic load such as the lower yield production [Glick, 1995].

These studies have been later reinforced and expanded, for example thanks to the studies like the one led by Jaramillo and coworkers in which they modeled and experimentally proved how heterologous expression affects cell's behavior [Carrera et al., 2011]. They studied how this heterologous expression conditions cell's growth rate in different strains and with different media, pointing out to the ribosomes as a limiting source. It is worth noting here that Jaramillo and colleagues use the term *Genetic Load* to refer to the *"fitness reduction at the expense of heterologous system expression"*. This term was indeed also used in a similar context

by Tabor and colleagues a few years before while relating the presence of large circuits with the metabolic burden phenomenon[4] [Tabor et al., 2009].

Later on, Cardinale and colleagues further contributed to understand the role of the cells genetic background in this process by studying different strains of *E. coli* [Cardinale et al., 2013, Arkin, 2013]. Other complementary studies at that time stressed the role of molecular competition [De Vos et al., 2011] and of chassis genome organization [Danchin, 2012]. Finally, this last year the groups led by DelVecchio at MIT and Ellis at the Imperial College have published interesting works that shed more light on this issue and contribute to reinforce our findings [Ceroni et al., 2015, Gyorgy et al., 2015]. The differences and coincidences with these two works will be further discussed below.

Overall, our work presented here is framed in a set of recent theoretical and experimental studies that establish the competition for a limited amount of cellular resources and machinery for gene expression and cell growth as one of the main context-related mechanisms explaining the interaction between synthetic circuits and its hosts [Klumpp et al., 2009, Scott et al., 2010, Weiße et al., 2015, Algar et al., 2014, Ceroni et al., 2015, Gyorgy et al., 2015].

The first of these works showed how gene expression was affected by growth rate-dependent parameters such as plasmid copy number, RNA polymerase (*RNAP*), ribosomes and even the growth medium, showing an excellent agreement between model prediction and experimental data of an unregulated gen [Klumpp et al., 2009]. This work led by Hwa already insinuated a linear correlation between gene expression and growth-rate that was backed up by the results later obtained again by Hwa's group [Scott et al., 2010]. Interstingly, as in our mathematical framework, yet with less emphasis and explicit description, they suggested a parallelism linking this 'Growth Laws' with the Ohm's law of electricity. In their

[4]Disambiguation: it should be clarified that this concept is also used in the field of *Population Genetics*, where it has a slightly different meaning, referring to the reduction of fitness of a population with a given genotype as compared to that of the optimum genotype [O'Donald, 1967]

opinion, this analogy could *"facilitate our understanding of the operation and design of complex biological systems well before all the underlying regulatory circuits are elucidated at the molecular level"*, as it happened with electricity before having full microscopic knowledge. These two previous works linked cells physiological state with (heterologous) gene expression in a general way. Weiße and colleagues have recently gone further following this line by exploring in more deep detail how three constrains or trade-offs related to cell physiological state may condition gene expression, namely, cellular energy, free ribosomes and proteins [Weiße et al., 2015]. Their model effectively couples gene expression with growth rate and growing populations, thus connecting molecular mechanisms to cellular behavior and providing more insight into host-circuit interactions.

All these papers serve as a precedent for our work presented here. However, they mainly focus on relating gene expression with cell behavior (i.e. growth), instead of on linking the expression of different genes within a cell, as in our case. Similar models made on this basis have also been recently developed, as already mentioned, by the groups led by Ellis and DelVecchio. These models explicitly consider only the process of translation [Algar et al., 2013, Algar et al., 2014] or both transcription and translation [Gyorgy and Vecchio, 2014, Vecchio and Murray, 2014]. All of them also show how competition for the shared limited amount of resources between the host and synthetic circuits is a mechanism able to couple *a priori* unconnected or unregulated genes (e.g. constitutive). Our work is closely related to their efforts to show these effects of shared resources on gene expression. As in our case, both groups have recently further complemented their models with experimental evidences that support and reinforce our hypotheses [Ceroni et al., 2015, Gyorgy et al., 2015].

On one hand, Ellis and colleagues developed an *in vivo* capacity monitor to constantly follow the effects that the expression of other unrelated genes, via plasmid for example, could have on the capacity monitor (expected to be constant). This strategy allowed them to show that changes in gene expression preceded those in growth rate, showing how depletion of resources due to consumption by other (synthetic) sources is what leads to a decreased growth. Furthermore, they studied the effects of modify-

ing the load of synthetic circuits through tuning translation (e.g. *RBSs*) and highlighted, following previous studies, how the free ribosome pool is a critical factor influencing gene expression and cell growth. On the other hand, DelVecchio and her group implemented a model accounting for competition for transcriptional (i.e. *RNAP*) and traslational (i.e. ribosomes) machinery. We have all converged in a parallel way to similar findings but there are also notable differences. Now, after having contextualized the topic, I am going to discuss our results and contrast, compare and complement them with theirs.

Our theoretical framework assumes limitation of machinery and resources both at the level of transcription and translation, including *RNAP*, ribosomes and related factors. It describes how a process of competition for these resources emerges between the different genetic demands (i.e. genetic loads), thus introducing a higher level of dependencies (i.e. coupling) between such demands or loads, even though they share no direct regulation between them (e.g. constitutive genes). As a result, *a priori* orthogonal and unconnected modules or devices become coupled because they share the same host and its resources for gene expression. As commented in the beginning of this section, this situation acts against the assumption of modularity and therefore undermines our capacity to properly and reliably predict the outcome of our designs if all these issues are not taken into account. Hence, our theoretical framework and the insights it provides should help to set improved guidelines for the design and construction of Synthetic Biology devices.

The mathematical formula of our framework for the expression of the different genetic demands is fairly similar to the Ohm's law governing electric circuit's behavior. In this analogy, the different genetic loads are associated with different resistances in circuits in a series and the voltage is equivalent to the measured fluorescence. This is a notable finding of our work: the behavior of genetic loads within cells is comparable to the behavior of resistances connected in a series to a real power supply in any electric circuits. This convergence is not by chance but is based on the same physical principle: in electric circuits there is competition for electric charges as in genetic circuits there is competition for cellular

machinery and resources.

As a result of this, the total genetic load of the cell is the sum of all genetic loads associated to each foreign gen involved in the synthetic device and of the rest of the cells inherent genetic loads. However, the capacity of a given cell to support increasing genetic loads is limited. One part of the cellular resources should be devoted to the maintenance of the regular cellular processes, which represent an internal genetic load that is determined by the cells genetic background. This is fairly similar to the internal resistance of a real power supply in electric circuits. The other part of the resources should be used to express the foreign genes of the synthetic circuit, which in turn represent the electric resistances connected in a series to such power supply.

Consequently, the behavior of the cell in response to increasing loads matches that of a power supply with increasing electric loads. Our model and its experimental validation reveal the existence of two different regimes. The first, where gene expression increases linearly when genetic load increases, and the second, where this dependence is sub-linear. This behaviour is also observed in electric circuits. The limits between both regimes depend on the values of the internal genetic load, as in electric circuits does with respect to the internal resistance of the power supply. Thus, the bigger the genetic background of the cell (or internal resistance of the real power supply), the bigger the range within which the cell supports linear behavior in response to increasing genetic loads before entering to the sub-linear regime. Noteworthy, this additive property of genetic circuits, i.e. the total genetic load is the sum of the genetic loads associated to each gene, and the convergence between electric and genetic systems, is very relevant in terms of predictability and it is not captured by other models such as DelVecchio's one.

Another interesting scenario we explored was how this unintended coupling between different genetic loads occurred. We employed and experimental an conceptual set-up that is similar to the one used by DelVecchio and colleagues and also to the one implemented by Ellis and his group. It consists of two orthogonal reporter systems: one constitutive, which acts as a monitor and should remain constant, and another one tun-

able (e.g. via promoter or RBS strength, gene copy number, etc). Hence, the effects of modifying the load of the tunable gene can be captured by the monitor one. Noteworthy, the coupling of both systems follows a linear relation with strong agreement between model predictions and experimental results, even with several tuning strategies through a number of key parameters and in different genetic implementations. This relationship is in our case captured by a formalism fairly similar to the Ohm's law, whereas in the case of DelVecchio's group they use an analogy borrowed from microeconomics theory and describe the linear relationship as isocost lines[5].

Some of the concerns about a possible bias in the results because of an unexpected coupling of the reporters due to its inherent nature or because they may interact with other expressed proteins, like for instance LuxR, were discarded in their control experiments. Despite swapping the reporters, changing one of them for another protein or even deleting one of them, the linear relation was maintained. In their work, DelVecchio and colleagues briefly mention a decline in growth rates when the expression of the tunable system is increased, which is consistent with previous works already cited. In our study, despite the fact that we did not explicitly include these measurements, growth rate decrease after gene expression induction was also observed though not quantified.

Regarding the main differences between our works, they suggest the existence of a separation of the pool of resources available for plasmid genes expression and for genomic genes expression. Although they bring little experimental evidence to support this hypothesis, they speculate that the local depletion of resources might play a role in the extent of coupling among gene expression levels of different genes. Further investigation on the effects of spatial proximity in the coupling of gene expression levels due to resource sharing are not included in their work. Our model does not contemplate this possibility. Indeed it is worth stressing that in their paper they comment that this 'separation' should not affect the existence of the linear dependence, but only the extent of coupling.

[5]In microeconomics, isocost lines describe the relation established between different products when there is a limited budget

Overall, the analogy of the Ohm's law serves a good guidance to describe our systems in terms of non-interacting entities competing for a common supply of resources. Interestingly, as mentioned before, it was also used to describe the relationship between gene expression and growth rate [Scott et al., 2010]. Furthermore, Ohm's law has also shown to be a valid analogy for other kind of systems like different transportation systems [Akers et al., 2006, Guyton and Hall, 2006, Sharpeshkar, 2010] and is therefore not circumscribed to genetic and electric systems. All these things suggest the existence of a more fundamental principle that may emerge in systems where there is competition for shared limited resources needed to perform different activities of regulation.

Our model provides a good qualitative understanding of the mechanism driving genetic behavior that goes in the same line of other works recently published. Moreover, it offers a quantitative approach for estimating the relative load of the different genetic loads within a cell. This approach might complement other strategies that are being implemented to deal with the load and other contextual issues that prevent or difficult the modular design of biological devices. Good examples are the development of promoter insulators [Davis et al., 2011]; the use of module insulators (or buffers) for reducing or preventing retroactivity effects [Del Vecchio et al., 2008, Del Vecchio, 2015]; the implementation of orthogonal cell machinery for gene expression in order to avoid interference due to competition [Wang et al., 2013]; the widespread quantification and characterization of collections of elements across different contexts [Lucks et al., 2008, Mutalik et al., 2013a, Mutalik et al., 2013b]; the use of load drivers to mitigate the impact of load on circuit function [Mishra et al., 2014, Klavins, 2014]; or the redesign of circuits to reduce its burden [Ceroni et al., 2015], among others.

Finally, it would be interesting to check whether the Ohm's law for assessing genetic load in bacteria also applies to other more complex organisms. Previous evidence found in eukaryotic systems suggest that gene expression may also be affected by the genetic load [Yallop and Svendsen, 2001b, Yallop and Svendsen, 2001a, van Rensburg et al., 2012]. If this is the case, this would reinforce the 'universality' of our findings. In

the case that it does not, it could point out other levels and mechanisms of regulation. Moreover, this law may contribute to the definition of new metrics that should allow us to quantify the genetic load of a given circuit and predict whether it will work or not in a cell, alone or combined with other circuits. Further work these directions should be devoted.

5.4 Final considerations

Throughout this Thesis we have reviewed the approaches taken until the present time to overcome the factors that still hinder the complete unfolding of Synthetic Biology as a true engineering discipline. The current theoretical and conceptual corpus has proved insufficient. Our aim within this Thesis has been to help to develop better and more robust theoretical foundations for a reliable and predictable design of synthetic devices. Furthermore, and following the credo of the field "making as knowing", our intention has also been to shed some light on the underlying principles that govern living systems during the process.

I am aware of the high level of difficulty that these goals entail. For me, it is still an open and legitimate debate whether Synthetic Biology will finally become, sooner or later, a full member of the 'Engineering club'. Either way, I think that the tension between 'making as knowing' and classical scientific approaches is productive, both in terms of the applications and solutions that might be developed during the process and of the knowledge that can be meanwhile uncovered.

* * *

Last but not least, I would like to connect with the reflections exposed in the *Preface*. During these almost five years of my PhD venture I have had the opportunity to learn a lot, at many different levels. Regarding Science, I have tasted its beauty but I have also seen how it is too often blurred -or distorted- by personal and economic interests. When it comes to a discipline with so much potentially transforming power, as Synthetic Biology has, I think that it is imperative to bring it as close as possible to the democratic and transparent practices oriented towards the common good that should pervade all societies. It is of utmost importance to put reason and science at the service of the fights and efforts devoted to preserve our planet and eradicate social inequalities and all the dangers that threaten our existence, especially of those who belong to the 99 per cent.

Chapter 6

CONCLUSIONS

After all I have exposed and discussed, the objectives that were initially set are again listed below followed by their corresponding **conclusions**:

O.1. Explore the field of Synthetic Biology and how it unfolds as an engineering discipline.

- Synthetic Biology, which results from bringing together biology and engineering, is still far from being considered as even a slightly mature engineering discipline.
- The development of solid and consolidated principles for engineering living systems is a challenging cornerstone of the field that should help to unlock the full potential of Synthetic Biology.
- An hybrid approximation that is able to fully exploit and combine the power of theoretical and experimental approaches have revealed as an optimal methodology to achieve this goal.

O.2. Explore how to improve standardization via better characterization of the synthetic devices.

- The use of an enzymology-based approach provides a framework for the study and reliable characterization of synthetic devices uncovering interesting connections of the principles of organization of natural systems.
- This framework offers causal connections between the experimental data obtained with the transfer function and the underlying biological mechanisms and questions the suitability of the Hill function beyond that of merely fitting experimental results.
- In our case of study, the Lux-inducible system, the affinity of the sensor device can be shifted by varying the amount of receptor through RBS strength tuning.

CONCLUSIONS

- Our model and the experimental evidences support previous findings that question the need for cooperativity in such systems.

O.3. Explore circuit-host interactions and its relation with the lack of proper modularity and the metabolic load.

- Our results show how *a priori* orthologous genes are indirectly coupled through its interaction with the host due to the competition of the different coexisting genetic loads for the limited amount of cellular resources and machinery.
- Our mathematical framework converges with Ohm's law showing that genetic loads within a cell behave similarly to electric loads connected in a series to a real power supply.
- The cell's genetic background plays an important role in determining the range in which the response of the cell to increasing the genetic loads is linear, in a parallel way to the internal resistance in electric circuits.
- The characteristics of the linear relation that is established between two genetic loads depends on the specific genetic characteristics of the device but also on the genetic background of the host cell.

Chapter 7

ANNEX

Synthetic Collective Intelligence

I spent my first years of the PhD working on the project that, until the moment, has culminated with the publication of the paper whose first page is attached in the following page [Solé et al., 2016]. The idea, as the reader shall find in the paper, was to try to turn bacteria into Swarm Intelligent Systems, like ants or termites, and achieve something that could be labeled as Synthetic Swarm Intelligence.

Swarm Intelligent Systems are able to perform surprisingly complex behaviors despite the simplicity -limited cognitive abilities- of the individuals (or agents) composing the systems. This is thought to happen mainly due to the ways in which the 'agents' communicate with each other, like implementing feedback loops, what might lead to the emergence of quite complex phenomena like symmetry-breaking scenarios or collective oscillations.

Computer simulations and robots have been the ways in which these kind of systems and their behavior have been approached so far, besides the classical biological approximation to such natural systems (i.e. study of organisms ecology, physiology, and so on). The use of Synthetic Biology to genetically engineer unicellular systems (e.g. bacterial cells) to make them display behaviors typical of swarm intelligent systems is a novel and challenging approach.

The paper that is attached here and in which I participated serves to delineate some possible ways in which this goal could be achieved. It proposes some genetic architectures and circuits and provides experimental models of bacteria implementing them and displaying swarm-intelligent-like behavior.

While I was working in this project, besides working on the modeling branch, I was trying to implement such circuits *in vivo* with bacterial cells. Although we had some good results that have not yet been published, we find several difficulties that hindered our experimental progress in this project and shifted our focus to some basic problems inherent to the fact of engineering synthetic circuits for its implementation within living cells.

Solé R, Amor DR, Duran-Nebreda S, Conde-Pueyo N, Carbonell-Ballestero M, Montañez R. Synthetic collective intelligence. Biosystems. 2016 Feb 8. pii: S0303-2647(16)30002-8. doi: 10.1016/j.biosystems.2016.01.002

Bibliography

[Akers et al., 2006] Akers, A., Gassman, M., and Smith, R. (2006). *Hydraulic Power System Analysis*. Taylor and Francis.

[Algar et al., 2014] Algar, R., Ellis, T., and Stan, G. (2014). Modelling essential interactions between synthetic genes and their chassis cell. In *CDC*, pages 5437–5444.

[Algar et al., 2013] Algar, R., Ellis, T., and Stan, G.-B. (2013). Modelling the burden caused by gene expression: an in silico investigation into the interactions between synthetic gene circuits and their chassis cell. *arXiv preprint arXiv:1309.7798*.

[Alon, 2006] Alon, U. (2006). *An introduction to systems biology: design principles of biological circuits*. CRC press.

[Andrianantoandro et al., 2006] Andrianantoandro, E., Basu, S., Karig, D. K., and Weiss, R. (2006). Synthetic biology: new engineering rules for an emerging discipline. *Molecular systems biology*, 2(1).

[Ang et al., 2013] Ang, J., Harris, E., Hussey, B. J., Kil, R., and McMillen, D. R. (2013). Tuning response curves for synthetic biology. *ACS Synthetic Biology*, 2(10):547–567.

[Arkin, 2008] Arkin, A. (2008). Setting the standard in synthetic biology. *Nature biotechnology*, 26(7):771–774.

[Arkin, 2013] Arkin, A. P. (2013). A wise consistency: engineering biology for conformity, reliability, predictability. *Current opinion in chemical biology*, 17(6):893–901.

[Arkin and Fletcher, 2006] Arkin, A. P. and Fletcher, D. a. (2006). Fast, cheap and somewhat in control. *Genome biology*, 7(8):114.

[Arpino et al., 2013] Arpino, J. A., Hancock, E. J., Anderson, J., Barahona, M., Stan, G.-B. V., Papachristodoulou, A., and Polizzi, K. (2013). Tuning the dials of synthetic biology. *Microbiology*, 159(7):1236–1253.

BIBLIOGRAPHY

[Ausländer et al., 2012] Ausländer, S., Ausländer, D., Müller, M., Wieland, M., and Fussenegger, M. (2012). Programmable single-cell mammalian biocomputers. *Nature*, 487(7405):123–7.

[Bailey, 1993] Bailey, J. E. (1993). Host-vector interactions in Escherichia coli. *Advances in biochemical engineering/biotechnology*, 48:29–52.

[Baldwin et al., 2012] Baldwin, G., Bayer, T., Dickinson, R., Ellis, T., Freemont, P. S., Kitney, R. S., Polizzi, K., and Stan, G.-B. (2012). *Synthetic Biology - a primer*. Imperial College Press.

[Ball, 2011] Ball, P. (2011). *Unnatural: the heretical idea of making people*. Random House.

[Basu et al., 2005] Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., and Weiss, R. (2005). A synthetic multicellular system for programmed pattern formation. *Nature*, 434(7037):1130–4.

[Bayer and Smolke, 2005] Bayer, T. S. and Smolke, C. D. (2005). Programmable ligand-controlled riboregulators of eukaryotic gene expression. *Nature biotechnology*, 23(3):337–43.

[Beal, 2014] Beal, J. (2014). Bridging the gap: a roadmap to breaking the biological design barrier. *Frontiers in bioengineering and biotechnology*, 2.

[Benner and Sismour, 2005] Benner, S. a. and Sismour, a. M. (2005). Synthetic biology. *Nature reviews. Genetics*, 6(7):533–43.

[Bensaude Vincent, 2013] Bensaude Vincent, B. (2013). Discipline-building in synthetic biology. *Studies in History and Philosophy of Biological and Biomedical Sciences*, 44(2):122–129.

[Bentley et al., 1990] Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H., and Kompal a, D. S. (1990). Plasmid-encoded protein: The principal factor in the 'metabolic burden' associated with recombinant bacteria. *Biotechnology and Bioengineering*, 35(7):668–681.

[Bintu et al., 2005] Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J., and Phillips, R. (2005). Transcriptional regulation by the numbers: models. *Current opinion in genetics & development*, 15(2):116–24.

[Blount, 2015] Blount, Z. D. (2015). The unexhausted potential of e. coli. *Elife*, 4:e05826.

[Brophy and Voigt, 2014] Brophy, J. a. N. and Voigt, C. a. (2014). Principles of genetic circuit design. *Nature methods*, 11(5):508–20.

BIBLIOGRAPHY

[Bud, 1994] Bud, R. (1994). *The uses of life: a history of biotechnology*. Cambridge University Press.

[Cameron et al., 2014] Cameron, D. E., Bashor, C. J., and Collins, J. J. (2014). A brief history of synthetic biology. *Nature reviews. Microbiology*, 12(5):381–90.

[Canton et al., 2008] Canton, B., Labno, A., and Endy, D. (2008). Refinement and standardization of synthetic biological parts and devices. *Nature biotechnology*, 26(7):787–93.

[Carbonell-Ballestero et al., 2014] Carbonell-Ballestero, M., Duran-Nebreda, S., Montanez, R., Sole, R., Macia, J., and Rodriguez-Caso, C. (2014). A bottom-up characterization of transfer functions for synthetic biology designs: lessons from enzymology. *Nucleic Acids Research*, 42(22):14060–14069.

[Carbonell-Ballestero et al., 2016] Carbonell-Ballestero, M., Garcia-Ramallo, E., Montanez, R., Rodriguez-Caso, C., and Macia, J. (2016). Dealing with the genetic load in bacterial synthetic biology circuits : convergences with the Ohm ' s law. *Nucleic acids research*, 44(1):496–507.

[Cardinale and Arkin, 2012] Cardinale, S. and Arkin, A. P. (2012). Contextualizing context for synthetic biology - identifying causes of failure of synthetic biological systems. *Biotechnology Journal*, 7(7):856–866.

[Cardinale et al., 2013] Cardinale, S., Joachimiak, M. P., and Arkin, A. P. (2013). Effects of genetic variation on the e. coli host-circuit interface. *Cell reports*, 4(2):231–237.

[Carrera et al., 2011] Carrera, J., Rodrigo, G., Singh, V., Kirov, B., and Jaramillo, A. (2011). Empirical model and in vivo characterization of the bacterial response to synthetic gene expression show that ribosome allocation limits growth rate. *Biotechnology Journal*, 6(7):773–783.

[Ceroni et al., 2015] Ceroni, F., Algar, R., Stan, G.-B., and Ellis, T. (2015). Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nature methods*, 12(5):415–418.

[Chalfie et al., 1994] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, 263(5148):802–805.

[Chappell et al., 2015] Chappell, J., Watters, K. E., Takahashi, M. K., and Lucks, J. B. (2015). A renaissance in RNA synthetic biology: New mechanisms, applications and tools for the future. *Current Opinion in Chemical Biology*, 28:47–56.

[Chen and Silver, 2012] Chen, A. H. and Silver, P. A. (2012). Designing biological compartmentalization. *Trends in cell biology*, 22(12):662–670.

BIBLIOGRAPHY

[Chen et al., 1994] Chen, H., Bjerknes, M., Kumar, R., and Jay, E. (1994). Determination of the optimal aligned spacing between the Shine- Dalgarno sequence and the translation initiation codon of E.coli mRNAs. *Nucleic Acids Research*, 22(23):4953–4957.

[Church et al., 2014] Church, G. M., Elowitz, M. B., Smolke, C. D., Voigt, C. a., and Weiss, R. (2014). Realizing the potential of synthetic biology. *Nature reviews. Molecular cell biology*, 15(4):289–94.

[Cody et al., 1993] Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. (1993). Chemical structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein. *Biochemistry*, 32(1979):1212–1218.

[Cornish-Bowden, 2004] Cornish-Bowden, A. (2004). *Fundamentals of Enzyme Kinetics*. Portland Press, 3 edition.

[Cornish-Bowden, 2013] Cornish-Bowden, A. (2013). The origins of enzyme kinetics. *FEBS letters*, 587(17):2725–30.

[Crutzen and Stoermer, 2000] Crutzen, P. J. and Stoermer, E. F. (2000). The Anthropocene. *Global Change Newsletter*, 41:17–18.

[Csete and Doyle, 2002] Csete, M. E. and Doyle, J. C. (2002). Reverse Engineering of Biological Complexity. *Science*, 295:1664–1670.

[Danchin, 2012] Danchin, A. (2012). Scaling up synthetic biology: Do not forget the chassis. *FEBS Letters*, 586(15):2129–2137.

[Danino et al., 2010] Danino, T., Mondragón-Palomino, O., Tsimring, L., and Hasty, J. (2010). A synchronized quorum of genetic clocks. *Nature*, 463(7279):326–30.

[Davis et al., 2011] Davis, J. H., Rubin, A. J., and Sauer, R. T. (2011). Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Research*, 39(3):1131–1141.

[Davis et al., 2015] Davis, R. M., Muller, R. Y., and Haynes, K. A. (2015). Can the natural diversity of quorum-sensing advance synthetic biology? *Frontiers in bioengineering and biotechnology*, 3.

[De Vos et al., 2011] De Vos, D., Bruggeman, F. J., Westerhoff, H. V., and Bakker, B. M. (2011). How molecular competition influences fluxes in gene expression networks. *PloS one*, 6(12):e28494.

[Del Vecchio, 2015] Del Vecchio, D. (2015). Modularity, context-dependence, and insulation in engineered biological circuits. *Trends in biotechnology*, 33(2):111–9.

[Del Vecchio et al., 2008] Del Vecchio, D., Ninfa, A. J., and Sontag, E. D. (2008). Modular cell biology: retroactivity and insulation. *Molecular Systems Biology*, 4(161).

BIBLIOGRAPHY

[Di Ventura et al., 2006] Di Ventura, B., Lemerle, C., Michalodimitrakis, K., and Serrano, L. (2006). From in vivo to in silico biology and back. *Nature*, 443(7111):527–33.

[Ellis et al., 2011] Ellis, T., Adie, T., and Baldwin, G. S. (2011). DNA assembly for synthetic biology: from parts to pathways and beyond. *Integrative biology : quantitative biosciences from nano to macro*, 3(2):109–118.

[Ellis et al., 2009] Ellis, T., Wang, X., and Collins, J. J. (2009). Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nature biotechnology*, 27(5):465–71.

[Elowitz and Lim, 2010] Elowitz, M. and Lim, W. a. (2010). Build life to understand it. *Nature*, 468(7326):889–890.

[Elowitz and Leibler, 2000] Elowitz, M. B. and Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature*, 403(6767):335–8.

[Endy, 2005] Endy, D. (2005). Foundations for engineering biology. *Nature*, 438(7067):449–53.

[Engler et al., 2008] Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. *PloS one*, 3(11):e3647.

[Ferrell and Ha, 2014] Ferrell, J. E. and Ha, S. H. (2014). Ultrasensitivity part I: Michaelian responses and zero-order ultrasensitivity. *Trends in Biochemical Sciences*, 39(10):496–503.

[Frank, 2013] Frank, S. a. (2013). Input-output relations in biological systems: measurement, information and the Hill equation. *Biology direct*, 8(1):31.

[Gardner, 2013] Gardner, T. S. (2013). Synthetic biology: from hype to impact. *Trends in Biotechnology*, 31(3):123–125.

[Gardner et al., 2000] Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000). Construction of a genetic toggle switch in Escherichia coli. *Nature*, 403(6767):339–42.

[Gardner and Hawkins, 2013] Gardner, T. S. and Hawkins, K. (2013). Synthetic Biology: evolution or revolution? A co-founder's perspective. *Current Opinion in Chemical Biology*, 17(6):871–877.

[Garg et al., 2014] Garg, N., Manchanda, G., and Kumar, A. (2014). Bacterial quorum sensing: circuits and applications. *Antonie van Leeuwenhoek*, 105(2):289–305.

[Gibson et al., 2009] Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6(5):343–345.

BIBLIOGRAPHY

[Glick, 1995] Glick, B. R. (1995). Metabolic load and heterologous gene expression. *Biotechnology Advances*, 13(2):247–261.

[Goodman, 2008] Goodman, C. (2008). Engineering ingenuity at iGEM. *Nature chemical biology*, 4(1):13.

[Goutelle et al., 2008] Goutelle, S., Maurin, M., Rougier, F., Barbaut, X., Bourguignon, L., Ducher, M., and Maire, P. (2008). The hill equation: a review of its capabilities in pharmacological modelling. *Fundamental & clinical pharmacology*, 22(6):633–648.

[Gramelsberger, 2013] Gramelsberger, G. (2013). The simulation approach in synthetic biology. *Studies in history and philosophy of biological and biomedical sciences*, 44(2):150–7.

[Gruber and Gross, 2003] Gruber, T. M. and Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual review of microbiology*, 57:441–466.

[Grunberg and Serrano, 2010] Grunberg, R. and Serrano, L. (2010). Strategies for protein synthetic biology. *Nucleic Acids Research*, 38(8):2663–2675.

[Gustafsson and Vallverdú, 2015] Gustafsson, C. and Vallverdú, J. (2015). The Best Model of a Cat Is Several Cats. *Trends in Biotechnology*, 34(3):207–213.

[Guyton and Hall, 2006] Guyton, A. and Hall, J. (2006). *Textbook of Medical Physiology*. Elsevier Inc, 11 edition.

[Gyorgy et al., 2015] Gyorgy, A., Jiménez, J. I., Yazbek, J., Huang, H.-H., Chung, H., Weiss, R., and Del Vecchio, D. (2015). Isocost Lines Describe the Cellular Economy of Genetic Circuits. *Biophysical Journal*, 109(3):639–646.

[Gyorgy and Vecchio, 2014] Gyorgy, A. and Vecchio, D. D. (2014). Limitations and Trade-offs in Gene Expression due to Competition for Shared Cellular Resources. *Proc. IEEE Conf. on Decision and Control*.

[Hartwell et al., 1999] Hartwell, L., Hopfield, J., Leibler, S., and Murray, A. (1999). From molecular to modular cell biology. *Nature*, 402:C47–C52.

[Hasty et al., 2002] Hasty, J., McMillen, D., and Collins, J. J. (2002). Engineered gene circuits. *Nature*, 420(6912):224–30.

[Heinemann and Panke, 2006] Heinemann, M. and Panke, S. (2006). Synthetic biology–putting engineering into biology. *Bioinformatics (Oxford, England)*, 22(22):2790–9.

[iGEM, 2004] iGEM (2004). Registry of standard biological parts. 2016-02-09.

BIBLIOGRAPHY

[Isaacs et al., 2004] Isaacs, F. J., Dwyer, D. J., Ding, C., Pervouchine, D. D., Cantor, C. R., and Collins, J. J. (2004). Engineered riboregulators enable post-transcriptional control of gene expression. *Nature Biotechnology*, 22(7):841–847.

[Jackson et al., 1972] Jackson, D. a., Symons, R. H., and Berg, P. (1972). Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America*, 69(10):2904–2909.

[Jacob and Monod, 1961] Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of molecular biology*, 3(3):318–356.

[Jiang et al., 2013] Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology*, 31(3):233–239.

[Kastenhofer, 2013] Kastenhofer, K. (2013). Two sides of the same coin? The (techno)epistemic cultures of systems and synthetic biology. *Studies in History and Philosophy of Biological and Biomedical Sciences*, 44(2):130–140.

[Kaznessis, 2007] Kaznessis, Y. N. (2007). Models for synthetic biology. *BMC systems biology*, 1(1):47.

[Keller, 2009a] Keller, E. F. (2009a). Knowing As Making, Making As Knowing: The Many Lives of Synthetic Biology1. *Biological Theory*, 4(4):333–339.

[Keller, 2009b] Keller, E. F. (2009b). What Does Synthetic Biology Have to Do with Biology? *BioSocieties*, 4(2-3):291–302.

[Khalil and Collins, 2010] Khalil, A. and Collins, J. (2010). Synthetic biology: applications come of age. *Nature Reviews Genetics*, 11(5):367–379.

[Kitano, 2001] Kitano, H. (2001). *Foundations of Systems Biology*. The MIT Press.

[Kitano, 2002] Kitano, H. (2002). Systems biology: a brief overview. *Science*, 295(5560):1662–4.

[Kitney and Freemont, 2012] Kitney, R. and Freemont, P. (2012). Synthetic biology - The state of play. *FEBS Letters*, 586(15):2029–2036.

[Klavins, 2014] Klavins, E. (2014). Lightening the load in synthetic biology. *Nature biotechnology*, 32(12):1198–1200.

[Klumpp et al., 2009] Klumpp, S., Zhang, Z., and Hwa, T. (2009). Growth rate-dependent global effects on gene expression in bacteria. *Cell*, 139(7):1366–75.

[Knight, 2003] Knight, T. (2003). Idempotent Vector Design for Standard Assembly of Biobricks. *MIT Artificial Intelligence Laboratory*, pages 1–11.

BIBLIOGRAPHY

[Kosuri and Church, 2014] Kosuri, S. and Church, G. M. (2014). Large-scale de novo DNA synthesis: technologies and applications. *Nature Methods*, 11(5):499–507.

[Kramer et al., 2004a] Kramer, B. P., Fischer, C., and Fussenegger, M. (2004a). BioLogic gates enable logical transcription control in mammalian cells. *Biotechnol Bioeng*, 87(4):478–484.

[Kramer et al., 2004b] Kramer, B. P., Viretta, A. U., Baba, M. D.-E., Aubel, D., Weber, W., and Fussenegger, M. (2004b). An engineered epigenetic transgene switch in mammalian cells. *Nature Biotechnology*, 22(7):867–870.

[Krauss, 2010] Krauss, L. M. (2010). Life from a Test Tube? The Real Promise of Synthetic Biology. *Scientific American*.

[Kwok, 2010] Kwok, R. (2010). Five hard truths for synthetic biology. *Nature*, 463(7279):288.

[Le Novère, 2015] Le Novère, N. (2015). Quantitative and logic modelling of molecular and gene networks. *Nature reviews. Genetics*, 16(3):146–58.

[Levin-Karp et al., 2013] Levin-Karp, A., Barenholz, U., Bareia, T., Dayagi, M., Zelcbuch, L., Antonovsky, N., Noor, E., and Milo, R. (2013). Quantifying translational coupling in E. coli synthetic operons using RBS modulation and fluorescent reporters. *ACS Synthetic Biology*, 2(6):327–336.

[Lewontin, 2014] Lewontin, R. C. (2014). The New Synthetic Biology: Who Gains? *The NewYork Review of Books*, pages 1–7.

[Liang et al., 2011] Liang, J., Luo, Y., and Zhao, H. (2011). Synthetic biology: putting synthesis into biology. *Wiley interdisciplinary reviews. Systems biology and medicine*, 3(1):7–20.

[Lucks et al., 2008] Lucks, J. B., Qi, L., Whitaker, W. R., and Arkin, A. P. (2008). Toward scalable parts families for predictable design of biological circuits. *Current opinion in microbiology*, 11(6):567–73.

[Ma et al., 2012] Ma, S., Tang, N., and Tian, J. (2012). DNA synthesis, assembly and applications in synthetic biology. *Current Opinion in Chemical Biology*, 16(3-4):260–267.

[MacDonald et al., 2011] MacDonald, J. T., Barnes, C., Kitney, R. I., Freemont, P. S., and Stan, G.-B. V. (2011). Computational design approaches and tools for synthetic biology. *Integrative biology : quantitative biosciences from nano to macro*, 3(2):97–108.

[Macía et al., 2012] Macía, J., Posas, F., and Solé, R. V. (2012). Distributed computation: the new wave of synthetic biology devices. *Trends in Biotechnology*, 30(6):342–349.

BIBLIOGRAPHY

[Marchisio and Stelling, 2009] Marchisio, M. A. and Stelling, J. (2009). Computational design tools for synthetic biology. *Current opinion in biotechnology*, 20(4):479–485.

[Markoff, 2014] Markoff, J. (2014). A Synthetic Biology Conference Lures an Intriguing Audience. *The New York Times*, pages 3–6.

[Matsuoka et al., 2009] Matsuoka, Y., Ghosh, S., and Kitano, H. (2009). Consistent design schematics for biological systems: standardization of representation in biological engineering. *Journal of the Royal Society Interface*, 6(Suppl 4):S393.

[McGinn and Gut, 2013] McGinn, S. and Gut, I. G. (2013). DNA sequencing – spanning the generations. *New Biotechnology*, 30(4):366–372.

[Miller et al., 2012] Miller, M., Hafner, M., Sontag, E., Davidsohn, N., Subramanian, S., Purnick, P. E., Lauffenburger, D., and Weiss, R. (2012). Modular design of artificial tissue homeostasis: robust control through synthetic cellular heterogeneity. *PLoS Comput Biol*, 8(7):e1002579.

[Miller and Bassler, 2001] Miller, M. B. and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology*, 55(1):165–199.

[Mishra et al., 2014] Mishra, D., Rivera, P. M., Lin, A., Del Vecchio, D., and Weiss, R. (2014). A load driver device for engineering modularity in biological networks. *Nature biotechnology*, 32(12):1268–1275.

[Moon et al., 2012] Moon, T. S., Lou, C., Tamsir, A., Stanton, B. C., and Voigt, C. A. (2012). Genetic programs constructed from layered logic gates in single cells. *Nature*, 491(7423):249–253.

[Moore, 2006] Moore, G. (2006). Life 2.0. *The Economist*.

[Morange, 2009] Morange, M. (2009). A new revolution? the place of systems biology and synthetic biology in the history of biology. *EMBO reports*, 10(1S):S50–S53.

[Mutalik et al., 2013a] Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q.-A., Tran, A. B., Paull, M., Keasling, J. D., Arkin, A. P., and Endy, D. (2013a). Precise and reliable gene expression via standard transcription and translation initiation elements. *Nature methods*, 10(4):354–360.

[Mutalik et al., 2013b] Mutalik, V. K., Guimaraes, J. C., Cambray, G., Mai, Q.-A., Christoffersen, M. J., Martin, L., Yu, A., Lam, C., Rodriguez, C., Bennett, G., et al. (2013b). Quantitative estimation of activity and quality for collections of functional genetic elements. *Nature methods*, 10(4):347–353.

[Nilsson and A, 2011] Nilsson, J. W. and A, R. S. (2011). *Electric Circuits*. Prentice Hall, 9 edition.

BIBLIOGRAPHY

[O'Donald, 1967] O'Donald, P. (1967). Genetic load of an evolutionary change. *Nature*, 216(5122):1348.

[O'Malley, 2009] O'Malley, M. A. (2009). Making Knowledge in Synthetic Biology: Design Meets Kludge. *Biological Theory*, 4(4):378–389.

[Osterman et al., 2013] Osterman, I. A., Evfratov, S. A., Sergiev, P. V., and Dontsova, O. A. (2013). Comparison of mRNA features affecting translation initiation and reinitiation. *Nucleic Acids Research*, 41(1):474–486.

[Pasotti et al., 2012] Pasotti, L., Politi, N., Zucca, S., Cusella De Angelis, M. G., and Magni, P. (2012). Bottom-up engineering of biological systems through standard bricks: a modularity study on basic parts and devices. *PloS one*, 7(7):e39407.

[Peretti and Bailey, 1987] Peretti, S. W. and Bailey, J. E. (1987). Simulations of host-plasmid interactions in Escherichia coli: Copy number, promoter strength, and ribosome binding site strength effects on metabolic activity and plasmid gene expression. *Biotechnology and bioengineering*, 29(3):316–328.

[Porcar and Pereto, 2015] Porcar, M. and Pereto, J. (2015). Nature versus Design: Synthetic Biology or how to build a biological non-machine. *Integr. Biol.*

[Prindle and Hasty, 2012] Prindle, a. and Hasty, J. (2012). Making gene circuits sing. *Proceedings of the National Academy of Sciences*, 109(42):16758–16759.

[Purcell and Lu, 2014] Purcell, O. and Lu, T. K. (2014). Synthetic analog and digital circuits for cellular computation and memory. *Current Opinion in Biotechnology*, 29:146–155.

[Purnick and Weiss, 2009] Purnick, P. E. M. and Weiss, R. (2009). The second wave of synthetic biology: from modules to systems. *Nature reviews. Molecular cell biology*, 10(6):410–22.

[Qi et al., 2015] Qi, H., Li, B. Z., Zhang, W. Q., Liu, D., and Yuan, Y. J. (2015). Modularization of genetic elements promotes synthetic metabolic engineering. *Biotechnology Advances*, 33(7):1412–1419.

[Ramalho et al., 2016] Ramalho, T., Meyer, A., Mückl, A., Kapsner, K., Gerland, U., and Simmel, F. C. (2016). Single Cell Analysis of a Bacterial Sender-Receiver System. *Plos One*, 11(1):e0145829.

[Rasmussen et al., 2009] Rasmussen, S. et al. (2009). *Protocells: bridging nonliving and living matter*. The MIT Press.

[Ravasz et al., 2002] Ravasz, E., Somera, A. L., Mongru, D. A., Oltvai, Z. N., and Barabási, A.-L. (2002). Hierarchical Organization of Modularity in Metabolic Networks. *Science*, 297(5586):1551–1555.

BIBLIOGRAPHY

[Regot et al., 2011] Regot, S., Macia, J., Conde, N., Furukawa, K., Kjellén, J., Peeters, T., Hohmann, S., de Nadal, E., Posas, F., and Solé, R. (2011). Distributed biological computation with multicellular engineered networks. *Nature*, 469(7329):207–11.

[Rodriguez-Caso et al., 2005] Rodriguez-Caso, C., Medina, M. A., and Sole, R. V. (2005). Topology, tinkering and evolution of the human transcription factor network. *FEBS Journal*, 272(24):6423–6434.

[Sambrook and Russell, 2001] Sambrook, J. and Russell, D. (2001). *Molecular Cloning: A Laboratory Manual*. Number v. 1 in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

[Sanger et al., 1977] Sanger, F., Nicklen, S., and Coulson, a. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74(12):5463–7.

[Sardanyés et al., 2015] Sardanyés, J., Bonforti, A., Conde, N., Solé, R., and Macia, J. (2015). Computational implementation of a tunable multicellular memory circuit for engineered eukaryotic consortia. *Frontiers in physiology*, 6.

[Schyfter et al., 2013] Schyfter, P., Frow, E., and Calvert, J. (2013). Synthetic biology: making biology into an engineering discipline. *Engineering Studies*, 5(1):1–5.

[Scott et al., 2010] Scott, M., Gunderson, C. W., Mateescu, E. M., Zhang, Z., and Hwa, T. (2010). Interdependence of cell growth and gene expression: origins and consequences. *Science*, 330(6007):1099–1102.

[Serrano, 2007] Serrano, L. (2007). Synthetic biology: promises and challenges. *Molecular systems biology*, 3(1):158.

[Sharpeshkar, 2010] Sharpeshkar, R. (2010). *Ultra Low Power Bioelectronics*. Cambridge University Press.

[Slusarczyk et al., 2012] Slusarczyk, A. L., Lin, A., and Weiss, R. (2012). Foundations for the design and implementation of synthetic genetic circuits. *Nature reviews. Genetics*, 13(6):406–20.

[Smolke, 2009] Smolke, C. D. (2009). Building outside of the box: iGEM and the BioBricks Foundation. *Nature biotechnology*, 27(12):1099–102.

[Smolke and Silver, 2011] Smolke, C. D. and Silver, P. a. (2011). Informing biological design by integration of systems and synthetic biology. *Cell*, 144(6):855–859.

[Sobie et al., 2011] Sobie, E. A., Lee, Y.-S., Jenkins, S. L., and Iyengar, R. (2011). Systems biology—biomedical modeling. *Science signaling*, 4(190):tr2.

[Solé et al., 2016] Solé, R., Amor, D. R., Duran-Nebreda, S., Conde-Pueyo, N., Carbonell-Ballestero, M., and Montañez, R. (2016). Synthetic collective intelligence. *BioSystems*, pages pii: S0303–2647(16)30002–8.

BIBLIOGRAPHY

[Sprinzak and Elowitz, 2005] Sprinzak, D. and Elowitz, M. B. (2005). Reconstruction of genetic circuits. *Nature*, 438(7067):443–8.

[Tabor et al., 2009] Tabor, J. J., Salis, H. M., Simpson, Z. B., Chevalier, A. a., Levskaya, A., Marcotte, E. M., Voigt, C. a., and Ellington, A. D. (2009). A synthetic genetic edge detection program. *Cell*, 137(7):1272–81.

[Tigges et al., 2009] Tigges, M., Marquez-Lago, T. T., Stelling, J., and Fussenegger, M. (2009). A tunable synthetic mammalian oscillator. *Nature*, 457(7227):309–312.

[UNESCO, 2000] UNESCO (2000). The 20th century: 100 years of scientific creativity. Retrieved from: http://www.unesco.org/bpi/science/content/press/anglo/6.htm.

[van Rensburg et al., 2012] van Rensburg, E., Den Haan, R., Smith, J., van Zyl, W. H., and Görgens, J. F. (2012). The metabolic burden of cellulase expression by recombinant saccharomyces cerevisiae y294 in aerobic batch culture. *Applied microbiology and biotechnology*, 96(1):197–209.

[Vecchio and Murray, 2014] Vecchio, D. D. and Murray, R. M. (2014). *Biomolecular Feedback Systems Chapter 5*. Princeton University Press.

[Venturelli et al., 2015] Venturelli, O. S., Egbert, R. G., and Arkin, A. P. (2015). Towards engineering biological systems in a broader context. *Journal of molecular biology*, 428(5B):928–944.

[Vilanova and Porcar, 2014] Vilanova, C. and Porcar, M. (2014). iGEM 2.0—refoundations for engineering biology. *Nature Biotechnology*, 32(5):420–424.

[Vilanova et al., 2015] Vilanova, C., Tanner, K., Dorado-Morales, P., Villaescusa, P., Chugani, D., Frías, A., Segredo, E., Molero, X., Fritschi, M., Morales, L., et al. (2015). Standards not that standard. *Journal of biological engineering*, 9(1):1.

[Voigt, 2006] Voigt, C. a. (2006). Genetic parts to program bacteria. *Current opinion in biotechnology*, 17(5):548–57.

[Wang et al., 2013] Wang, Y.-H., Wei, K. Y., and Smolke, C. D. (2013). Synthetic biology: advancing the design of diverse genetic systems. *Annual review of chemical and biomolecular engineering*, 4:69.

[Watson and Crick, 1953] Watson, J. D. and Crick, F. H. C. (1953). Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature*, 171:737–738.

[Way et al., 2014] Way, J. C., Collins, J. J., Keasling, J. D., and Silver, P. A. (2014). Integrating biological redesign: Where synthetic biology came from and where it needs to go. *Cell*, 157(1):151–161.

[Weiss and Knight, 2000] Weiss, R. and Knight, T. (2000). *Lecture Notes in Computer Science*, volume 7246. Springer.

BIBLIOGRAPHY

[Weiße et al., 2015] Weiße, A. Y., Oyarzún, D. A., Danos, V., and Swain, P. S. (2015). Mechanistic links between cellular trade-offs, gene expression, and growth. *Proceedings of the National Academy of Sciences of the United States of America*, 112(9):E1038–1047.

[Westerhoff and Palsson, 2004] Westerhoff, H. V. and Palsson, B. O. (2004). The evolution of molecular biology into systems biology. *Nature Biotech*, 22:1249–1252.

[Wikipedia, 2016] Wikipedia (2016). Synthetic biology. Online; accessed 04-March-2016.

[Yallop and Svendsen, 2001a] Yallop, C. and Svendsen, I. (2001a). *Recombinant Protein Production with Prokaryotic and Eukaryotic Cells. A Comparative View on Host Physiology*. Springer.

[Yallop and Svendsen, 2001b] Yallop, C. A. and Svendsen, I. (2001b). The effects of G418 on the growth and metabolism of recombinant mammalian cell lines. *Cytotechnology*, 35(2):101–114.

[Yao et al., 2013] Yao, A. I., Fenton, T. A., Owsley, K., Seitzer, P., Larsen, D. J., Sit, H., Lau, J., Nair, A., Tantiongloc, J., Tagkopoulos, I., and Facciotti, M. T. (2013). Promoter element arising from the fusion of standard BioBrick parts. *ACS Synthetic Biology*, 2(2):111–120.

[Yeh and Lim, 2007] Yeh, B. J. and Lim, W. A. (2007). Synthetic biology: lessons from the history of synthetic organic chemistry. *Nature Chemical Biology*, 3(9):521–525.

[Zhang et al., 2015] Zhang, C., Tsoi, R., and You, L. (2015). Addressing biological uncertainties in engineering gene circuits. *Integrative biology : quantitative biosciences from nano to macro*.

[Zheng and Sriram, 2010] Zheng, Y. and Sriram, G. (2010). Mathematical modeling: bridging the gap between concept and realization in synthetic biology. *BioMed Research International*, 2010.