Genetically Engineering Living Systems

From complex computational scenarios to múltiple market applications

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“The time has come for synthetic biologists to develop more real-world applications. The field has had its hype phase, now it needs to deliver”

Christopher A. Voigt

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ABSTRACT

Nowadays, the possibility to construct living technologies has open a broad range of opportunities to tackle society-impact problems. In this PhD thesis, we have fused scientific disciplines to re-design methodologies and bring new close to market solutions.

First, we have established a new framework for circuit design, by creating simple, independent, and functional modules which can be interconnected with diffusible molecules using 2D surfaces, and which can perform any type of computation based on its specific spatial arrangement.

Next, we have explored the wide versatility of elements present in nature for detection purposes. Thus, we have developed two proof of concept biosensor designs. The first one uses diffusion patterning for visual quantification of chemical compounds as a screening method, whereas the other one is based on the detection of pathogenic microorganisms through their quorum sensing fingerprints to build a continuous monitoring system to anticipate infections.

Actualment, la possibilitat d'obtenir tecnologies vives ha obert un ventall de possibilitats per tal d'atacar problemes de gran impacte en la nostra societat. En aquesta tesi doctoral, s'han fusionat varies disciplines per redissenyar metodologies i oferir solucions més properes al mercat.

En el primer treball, s'ha explorat un nou paradigma per al disseny de circuit sintètics, a través de la interconnexió d'elements computacionalment actius en superfícies 2D gràcies a molècules difusores. Això permeten obtenir qualsevol tipus de circuit en funció de la disposició espacial.

El segon treball fa èmfasi en els biosensors. Primerament s'explora una metodologia per crear biosensors per a la detecció i quantificació visual de substàncies químiques a través de diferències en patrons de difusió. Seguidament, s'explora una nova vertical de sensors per a agent microbiològics que té com a finalitat la seva monitorització en continu gràcies al patró de secreció de molècules quorum sensing, anticipant futures infeccions.

PREFACE

Living organism can be understood as highly organized entities that can react to stimuli, reproduce, grow, adapt, and maintain homeostasis[1]. Many of these functions are executed by molecular machines, which consume energy and convert it into mechanical work[2]. The promise of building living synthetic machines with a pre-defined topology and functionality give us the chance to implement rational designs with specific purposes to mimic the *in vivo* counterparts[3].

In physics, systems that appear extremely complex often can be described by simple laws. Biological systems have resisted such abstractions, not because they are inherently more complex, but because their structure and behaviour are described by a genome, which is itself a store of information[4]. Nevertheless, this information can be interpreted at different levels of abstraction, going from specific codons which codify specific amino acids in the DNA to general processes or behaviours[5].

For instance, Synthetic biology is a field of research in which the main objective is to create fully operational biological systems from the smallest constituent parts possible, including DNA, proteins, and other organic molecules. This discipline rationalizes this complex living organisms by considering them as machines which receive information, process it, and use this information to decide what will happen next[6–8].

This goal of redesigning organisms enables to obtain synthetic living machines which can produce a substance, such as a medicine or fuel, or gain a new ability, such as sensing something in the environment, among other functionalities[9,10].

However, issues such as obtaining well-characterized synthetic parts to give raise to these synthetic machines which can undertake complex, scalable, and robust behaviours remain a challenge. In this PhD thesis, we have analysed strategies for governing living organisms and making them perform complex computational operations from simple and standardized methodologies. As a first starting point, in Chapter 2 we have explored a more fundamental work, which takes inspiration from printed electronics and uses space as new computational element to achieve any type of circuit design which can perform either digital or analogue computations. From this first study, a more applied work has been carried in Chapter 3, which focuses on the

development of broad spectrum point of care biosensors, that can detect from chemicals compounds to pathogenic microorganism. The first section of this chapter uses knowledge from the previous work to standardize a way to construct inexpensive and scalable biosensors with visual readouts. These are single-use devices for the detection of chemical compounds, i.e., mercury as a proof of concept, which can be optimal for screening purposes. In the second section of this chapter, a different approach for biosensor design is explored, which makes use of quorum sensing secretion patterns for the identification and quantification of pathogenic microorganisms, i.e., focusing mainly on *Vibriosis*. In this case, a more sophisticated proof of concept design has been implemented with the final aim to obtain continuous and autonomous devices to monitor pathogen abundances and anticipate the appearance of infections. Moreover, findings from this chapter have been subject matter for a patent filling and its further commercialization through the creation of a spin-off company from the University Pompeu Fabra.

Differently from other thesis in biomedicine, I have included business aspects such as market exploration, regulatory and competitor landscape as fundamental concepts to take into consideration when trying to reach the market and bring a solution based on synthetic biology to society. These learning have been obtained from different accelerator programs.

The present PhD thesis has tested bacteria as the main model organism, and has fused multiple disciplines, going from mathematical models to describe systems, to business aspects to describe a business plan, stopping by taking electronic principles for circuit design and using engineering advances for device prototyping and using software for embedded programming and data analysis.

Living systems have a lot to offer and their potential is just about to be explored.

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CHAPTER 1

Introduction

Technological advances are a driving force for development and progress. While species in nature have instinctive behaviour, human beings possess highly developed capacities to think systematically to innovate and consciously modify the environment to adapt in rapidly changing circumstances.

By the early 20th century the term *technology* acquired a range of means, processes, and ideas, in addition to tools and machines. By mid-century technology was defined as "the means or activity by which man seeks to change or manipulate his environment[11]".

In the XXI century, problems are thought to be better tackled using living or hybrid technologies. The main challenge is to establish a standard methodology for the design of these new technologies, getting inspiration from already exiting inventions, as well as mature and well-established disciplines, such as electronics, mathematics, and chemistry[12,13].

Synthetic Biology offers innovative approaches for engineering new biological systems or re-designing existing ones. It has been described as a disruptive technology at the heart of the so-called Bioeconomy, with the capabilities of delivering new solutions to global healthcare, agriculture, manufacturing, and environmental challenges. However, despite successes in the production of some high value chemicals and drugs, there is a perception that synthetic biology is still not yet delivering all its potential[14,15].

The present PhD dissertation has a special focus on the progress of this highly ambitious discipline to meet current challenges, not only in biomedicine, but also in food and climate.

This thesis covers from basic and fundamental work to highly applied solutions to cover market needs. Nevertheless, all chapters try to display the high potential embedded in living systems and their vast applications in our society, considering living systems the next revolution[16].

1.1 Synthetic Biology Market

Synthetic Biology (SB) makes use of the richness of biological diversity to solve pressing, real-world problems. This includes the development of personalized medicines, energy-rich molecules for sustainable fuels, production of industrial products, remediation of polluted environments, and food supplies to meet the increasing global population demand. Although its great potential, we are still far from capitalizing all the benefits Synthetic Biology can bring[12,17,18].

The global SB market size is projected to reach USD 30.7 billion by 2026 from USD 9.5 billion in 2021, at a CAGR of 26.5% during the forecast period[19].

This massive growth of this sector is due to the variety of potential applications it has, the rising in R&D funding and methods which lower cost of DNA sequencing and synthesizing, as well as increased investments in the market. The US currently leads the world in intellectual advances, and commercial development.

However, in the US and worldwide, biosafety, biosecurity, and concerns regarding the lack of education on risks and benefits related to SB would hamper the growth of this market. Considering this lack of clarity at the governmental level, public perception is mixed, regarding genetically modified organisms (GMOs) and environmental and biosafety risks in our society. Thus, better communication and education are required to support the growth of SB research and products, especially those with a direct consumer connection[20].

It is a relatively new discipline as in 2003, fewer than ten articles in the scientific literature related to this field could be found. However, by 2011, that number grew to approximately 350, and nowadays, we can find thousands of them[21,22].

SB market can be divided into three segments: Enabling Technologies (basic technologies on which SB relies, e.g., DNA synthesis, sequencing, bioinformatics, data management tools); Core Technologies (those that define SB endeavours); and Enabled Products (products, services, or platforms resulting from or incorporating the end results of SB processes). These segments constitute a simple value chain[21].

1.2 Brief introduction to Synthetic Biology

The main goal of SB is to rationally engineer biological systems to generate tuneable and well-characterized parts and modules with different objectives to somehow modify cellular functions and responses[23,24]. By having rational and controllable logic elements, researchers can use living systems as engineered "biological machines" to perform a large range of functions[25]. In this context, biocomputation is the branch of Synthetic Biology aimed at implementing artificial computational devices using engineered biological motifs as building blocks.

The following image displays the design-build-test strategy with some of the newest advances on the field (Fig.1.1).

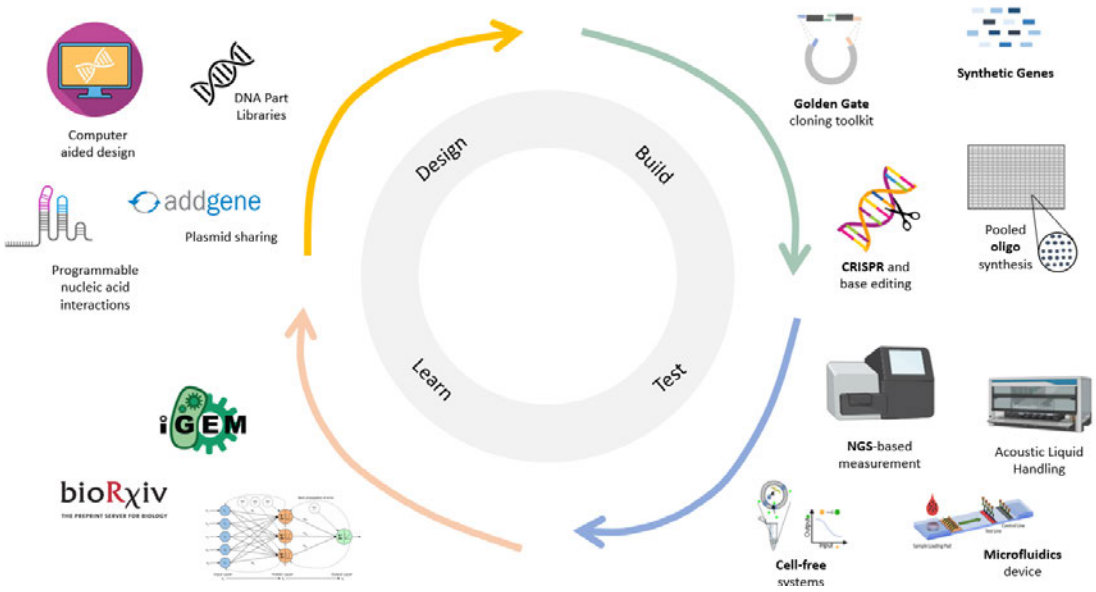


Figure 1.1. Design-build-test and tools in Synthetic Biology. From this workflow, four different steps can be observed: i) Design which implies modelling and experimental setups, ii) Build in regard to experimental assembly and the novel available techniques for its automation, iii) Test, to try what is being assembled with more innovative machinery, iv) Learn from the tests through data analysis, parameter obtention and re-designing, as well from the communities and the public sharing[26].

1.2.1 An emerging engineering-connected discipline

These machines need to be able to receive information, process it and use this information to decide what will happen next. Similarly, cells can sense inputs, process these inputs and make decisions to execute outputs[27].

In a computing device, inputs are mathematically processed into digital signals. For a binary code, the basic unit of information is denoted as a series of “0” and “1” digits, which represents two possible states in a logic circuit. These digital circuits use logic elements which can be interconnected to create logic gates. An important property of these logic gates is its ability to network together to implement more complex circuits[28]. Similarly, Synthetic Biology takes inspiration from these already established concepts, offering analogies of these digital signals based on presence or absence of specific molecules. Not surprisingly, most work in this field offers diagrams involving logic gates or computational circuits.

For this reason, Synthetic Biology is emerging as an engineering discipline which uses biological systems to perform this type of logics[15]. Nevertheless, many challenges need to be overcome due to the incomplete understanding of these living systems, the lack of tools for manipulating them and the unpredictability and leakage of the systems[29].

For instance, in biology, the complexity of a device grows together with the complexity of its genetics. Then, from these complex genetics many problems arise, such as the genetic load and the number of components being connected through different and orthogonal molecules, among others.

All these difficulties make hard to obtain trustable and reproducible devices that can work in multiple scenarios and that can be scalable for any industrial purpose or further biomedical applications.

Until now, improved methodologies have been implemented using multicellular consortia and spatial segregation as key points on living circuit designs. Thus, offering

components with low genetic engineering, which can be reused in different parts of the circuit and minimizing the wiring requirements to link all circuit components[30].

1.2.2 Synthetic Biology and Computational power

1.2.2.1 Multicellular consortia

Microbial consortia are widespread in nature, i.e., from the gut microbiome to soil microbial communities. In a consortium, not only competition among strains occur, but also cooperation and communication, allowing much complex behaviours[31]. It has been used in biotechnology, including fermentation, waste treatment, and agriculture.

Today, synthetic biologists are increasingly engineering microbial consortia for diverse applications, including bioproduction of medicines, biofuels, and biomaterials from inexpensive carbon sources. The better understanding of natural ecosystems will help develop tools to build synthetic consortia systems with pre-defined behaviours, which will improve the performance of the whole community.[32].

To harness these cooperative advantages in Synthetic Biology, different strategies such as cooperation between strains , selective environments, engineering self-limiting growth, and spatial separation of strains have been developed to keep cellular consortia stable[33].

When considering the development of complex computational circuits, cellular consortia play an important role, enabling to split different parts of the computation into different engineered cells. This can help designing complex decision-making biological circuits capable to cope with external signals and their changes[34].

The figure below (Fig.1.2) displays three different systems when consortia is used to achieve complex tasks.

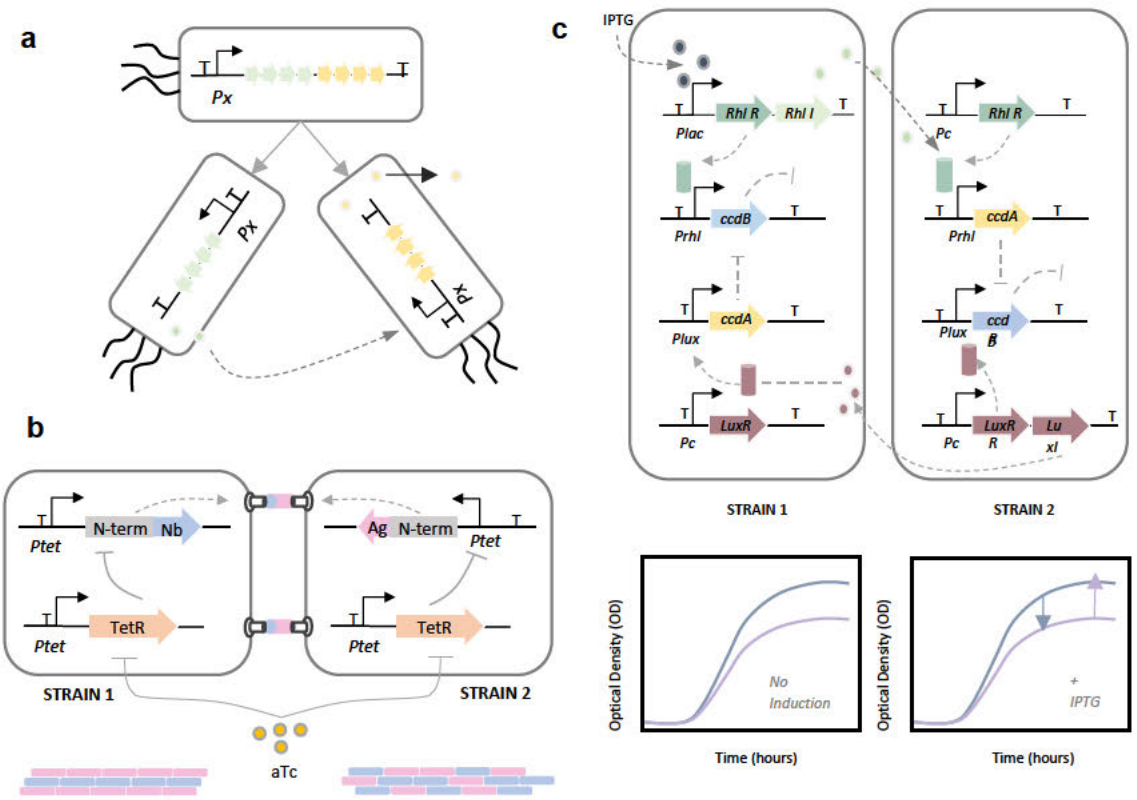


Figure 1.2. The use of synthetic microbial consortia. **a.** Pathway divided between member of the consortium through the creation of an intermediate metabolite. **b.** Spatial programming of cells by expressing a nanobody or an antigen respectively. Depending on an external input, i.e., anhydrotetracycline (aTc), different nanobody/antibody proteins can be used to form different patterns, as aTc acts by blocking cell-cell adhesion. **c.** The population can be regulated by genetic circuits and feedback, coupling together two quorum sensing systems with the repression of a toxin by an antitoxin[35].

1.2.2.2 Spatial segregation

A major endeavour in Synthetic Biology is to program functions of cells and cell populations in a predictable manner. It is critical for reaching the full potential of this discipline and translate it into different fields of application.

In nature, depending on environmental conditions, i.e., stress, nutrient, and temperature, many bacteria can form spatial structures forming cell aggregates, such as biofilms, which allow them to achieve functions beyond the capability of individual

cells or homogenous cell populations. This property has been considered a powerful design strategy to engineer sophisticated functions in engineered cell populations[36].

For instance, an additional gain in computational power is often achieved by the spatial arrangement of specific network components. Then, it is possible to create spatial compartments based on computational activators, which can interact with additional domains based on its spatial topology and computational behaviour. Figure 1.3 displays a theoretical example of this spatial segregation, which can allow greater computational power.

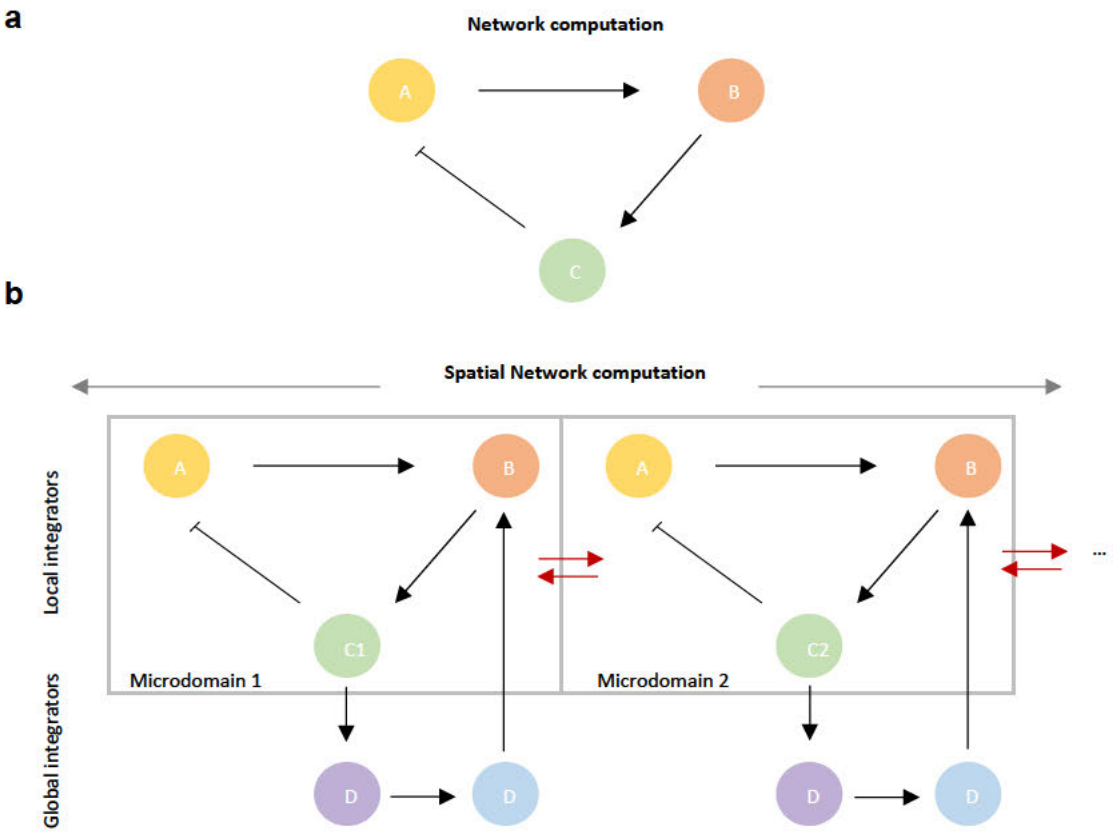


Figure 1.3. Spatial network computations. a. Network computation with no spatial component. **b.** Spatial computation segregation. Partitioning of the network to obtain two microdomains[37].

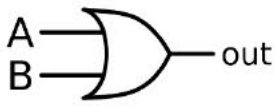
1.2.2.3 Synthetic circuits

The first synthetic circuits performing designed functions were implemented by modifying genetic regulatory circuits[38]. Nowadays, other approaches are explored, using alternative control points on transcription, translation, or post-translation level. These are employed by producing changes in DNA rearrangements, RNA manipulation, CRISPR-Cas9 technologies, MAPK signalling or cell-cell communication, among others.

Digital synthetic circuits use the concept of binary bits and take advantage of the extensive knowledge and success of digital circuits in electronics. In this case, circuits use biomolecules, whose amount or activity can switch from high "1" to low "0", as a response output (Fig. 1.4). Digital devices are made by combining single units, i.e., logic gates, and are expressed in a truth table (Fig. 1.4).

a

A	B	Out
0	0	0
0	1	1
1	0	1
1	1	1



b

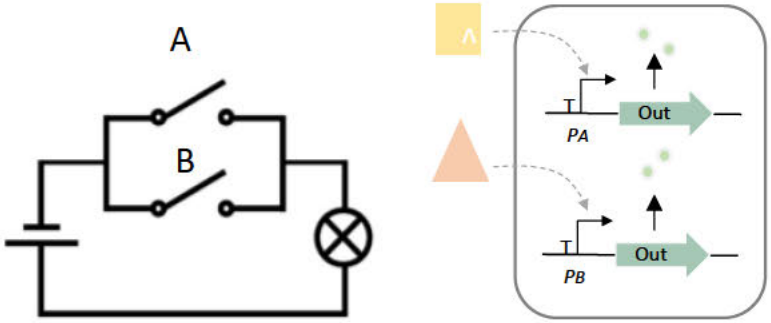


Figure 1.4. Synthetic circuit design. a. Truth table following an AND logic. **b.** System analogy between electronics and genetics to achieve the same computational behaviour.

Most genetic circuits in biology, are composed by a sensor able to respond to inputs, a computational module performing a desired computational behaviour and actuators to produce a proper output. To generate responsive pathways, many different inputs can be used, ranging from chemical molecules, i.e., arabinose, IPTG, to light, temperature or osmolarity.

A major challenge in the field of synthetic biology is the construction of complex logic circuits that analyse variables as in electronics; where a single circuit accepts one or more binary inputs to generate one or more binary outputs[39]. Some examples of genetic circuits mimicking computational behaviour are toggle switches, oscillators, Boolean logic gates, feedback controllers, and multiplexers. However, complex computational behaviours imply affections by gene expression noise, mutation, cell death, undefined, and changing extracellular environments and improper interactions with the cellular context [8]. Furthermore, complex genetic engineering is necessary when multiple input variables are analysed, limiting the processing capacity of the system.

The complexity of the genetic engineering required can be reduced by using distributed logic circuits, where the computation is distributed among several physically separated cellular consortia that each sense only one signal and respond by secreting a communication molecule[40]. Even though the consortium approach makes Boolean circuit design simpler, it still shows a slow response and considerable complexity since each cell needs to recognize, synthesize and secrete a wiring molecule.[41]

1.3 Applications in Synthetic Biology

1.3.1 Outside-the-lab overview

After introducing the multidisciplinary of this field, which takes advantage of engineering, physics, and chemistry, among other disciplines to enrich the know-how, a key aspect to achieve its full potential is to be successful bringing outside-the-lab solutions. To do so, systems should be genetically and functionally stable over long time periods and under variable storage conditions, as some solutions require high-tech machinery or high energy consumption system which are not optimal to reach certain market demands, i.e., storing conditions at -80°C for instance[42]. Other aspects to take into consideration are the need of minimal equipment and resources to run the solution, as well as minimal intervention by experienced professionals. In this regard, SB is shifting from utilizing biology to deploying biology[43].

To reach this market demand, Synthetic Biology approaches have been distinguished into two main groups, cell-based systems, and cell-free systems, both having some advantages and challenges associated[14,44].

In whole-cell platforms, mass-production is easier to be consolidated, as well as multiple complex reactions, being useful, for example, in the production of by-products in bioreactors. However, some challenges regarding long-term viability or stability, cell toxicity or time delays due to cell growth need to be faced[45].

On the other hand, in cell-free systems, cell viability is not yet a problem and manipulation are much easier, as there is no need to keep a living system. Although this system seems a better solution to be implemented out of the lab, problems such as batch variability, the short duration of cell-free reactions, the high costs of reagents to prepare extracts as well as difficulties to fold some complex protein products are also challenging these systems[46].

From these two approaches, we are introducing three major areas for outside-the-lab deployment of SB solutions: bioproduction, biosensing and living therapeutics and probiotics delivery.

1.3.2 Bioproduction

1.3.2.1 Cell-based systems

Synthetic Biology offers on-demand and continuous production of chemicals, therapeutics and food or food ingredients using different host organisms.

Genes codifying enzymes or synthetic pathways are increasingly being introduced into microbial hosts to produce value added chemicals from cheap sources[47].

Whole-cell solutions are the main production source of protein therapeutics, e.g. starting from of recombinant insulin in 1982. An early landmark achievement was the production of antimalarial Artimisin by Sanofi, although it was discontinued as some researchers managed to engineer *S cerevisiae* to produce the precursor for the antimalarial drug for a much cheaper price[18,48]. Other studies have explored the ability to engineer bacteria such as *P pastoris* to obtain a switchable production of two products of interest in a microfluidics reactor at single-dose level[49], as well as biofuel production using lignocellulose through engineered S cerevisiae[50], However, scalability, reaction time and availability of portable platforms are some limitations to be overcome.

1.3.2.2 Biotic/Abiotic Interfacing

To facilitate deployment of living systems, a special focus has been paced on coupling Material Sciences and Synthetic Biology to produce encapsulation-based platforms. Some studies have revealed the use of encapsuled Bacillus subtilis spores within 3D printed agarose hydrogels which can be induced to produce antibiotics. These spores are resilient to extreme environmental conditions and can work on-demand thanks to the induction capabilities[51]. Additionally, studies on compartmentalizing a consortium through spatial separation within printed hydrogels have also been studies, rather than co-culturing different strains to avoid competitiveness and facilitate control dynamics and protection in front of environmental perturbances[52].

1.3.2.3 Cell-free production platforms

As mentioned before, cell-free offers enhanced storage capabilities, as well as low-resources and potential remote use. Examples consist of freeze-dried extracts which can be rehydrated and produce functional products. Studies have revealed the capability to couple production and purification, i.e., chromatography, within the same device[53] or to express phages from cell-free transcription-translation platforms as a potential alternative to current antibiotic regimens[54]. Others have fused cell-free expression with microfluidics to create point-of-care cell-free production of therapeutics, offering a continuous production and purification to obtain antimicrobial doses within 6 hours[55]. Additionally, hosts are being explored to improve bioproduction efficiency, such as the use of the marine bacterium *Vibrio natriegens,* which has gained attention as an emerging microbial host for biotechnology due to its fast growth rate[56]. Some of the solutions for bioproduction are displayed in figure 1.5.

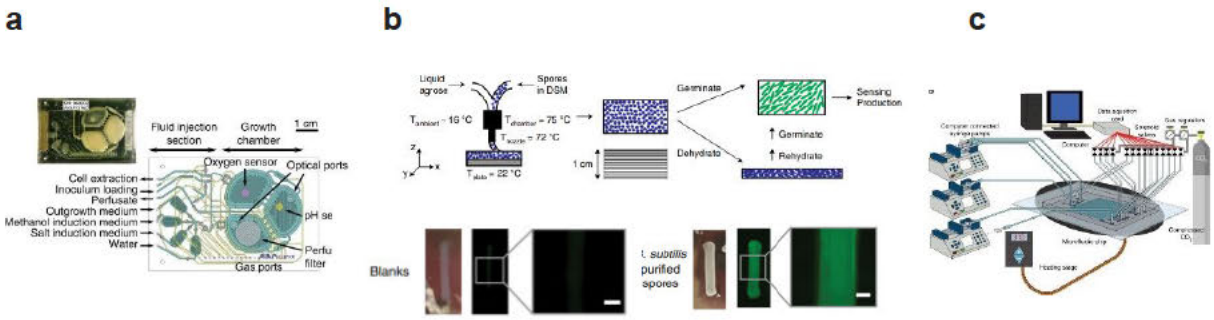


Figure 1.5. Bioproduction examples. a. Programmable protein production in a milliliter-scale microbioreactor. It is based on a microfluidic circuit that is equipped with a pneumatic routing of reagents, precise peristaltic injection, a growth chamber mixing and a fluid extraction[49]. **b.** 3D printer design and development of the bio ink. The printhead combines agarose and B. subtilis spores (blue circles). Below, experimental results display a comparison between a blank bar which does not have cells and B subtilis 168 bar which is constitutively expressing GFP[51]. **c.** Integrated cell-free system with computer-controlled solenoid valves and syringe pumps connected to a microfluidic device placed on a heating stage[55]

1.3.3 Biosensing

Biosensing has a broad range of applications and includes many different techniques, going from nucleic acids, i.e., PCRs, to whole-cell systems[57].

1.3.3.1 Cell-based biosensing platforms

Biosensing requires cell surveillance and rapid responses. Some explored strategies implied the use of biofilms to detect external signals and perform programmable responses benefiting from self-organized patterns [58]. Others have explored the use of bacteriophages due to its high specificity to target bacterial hosts[59,60]. Recently, these systems have been coupled to gold-binding peptides to display colorimetric aggregations[61].

Other promising technologies to offer point-of-care solutions integrate microfluidics or paper-based platforms for sensing purposes. Some examples include, microfluidic chips which allow sampling, incubation and photodetection in a fully automated device[62] or low-cost paper-based systems which enable the detection of bacterial contamination[63] or other molecules of interest, such as antibiotic resistances using β-galactosidase as the reporter protein[64].

1.3.3.2 Biotic/abiotic interfacing

Not surprisingly the already described 3D printing and embedded materials for cell storage are also useful in this field to enable long-term storage out of the lab. Some examples use biofilms or encapsulated spore-based platforms due to their resilience. For this reason, many groups have tried to mimic artificial spored using artificial 3D shells able to resist stressors, but also with the ability to degrade for cell-based actuation[65].

Strategies for these shells can range from the use of coatings based on metal-ligand complexes to encapsulation through alginate[66,67]. The combination of encapsulation with fluidics enables portable and easily deployable biosensing systems. Some examples include spore-based heavy metal whole-cell biosensor which was functionally stable for 1 year[68]. Another example is called SSHELs, a synthetic

bacterial spore-like particles, covalently covered with an affibody against HER2, a cell surface protein overexpressed in some breast and ovarian cancer cells[69].

Interfacing cells with microelectronics can widespread the potential, providing unique applications including hybrid bio semiconductor platforms. One recent advance includes an ingestible micro-bio-electronic device utilizing engineered probiotic *E. coli* NISSLE 1917 integrated with semiconductor microelectronics for *in situ* gastrointestinal biomarker detection via a bioluminescent detection circuit that communicates with an external device[70].

1.3.3.3 Cell-free biosensing platforms

Cell-free extracts can be better handled for on-site measurements. To enable this extract to work outside the lab in a friendly manner, some studies have developed paper-based freeze-died systems which can detect heavy-metal ions in water and quantify the response through a smartphone camera using light filters[71]. In other studies, researchers have engineered toehold switches to respond to saliva enriched with SARS-CoV-2 RNA, producing a bioluminescent protein[72].

Some groups have used advances in CRISPR/Cas-based systems as well as programmable RNA switches to create cell-free genetic circuits capable of discerning between mRNA from different Ebola strains[73] or to develop a rapid platform for sequence-specific Zika virus detection using an isothermal RNA amplification and CRISPR/Cas9-based analysis technique[74].

For rapid detection of nucleic acids, a Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), has been developed, involving recombinase-mediated polymerase pre-amplification of DNA or RNA, and subsequent Cas13- or Cas12-mediated detection via fluorescent and colorimetric readouts, providing results in less than 1 hour and detection at attomolar levels[75]. SHERLOCKv2 is an improved version which offers multi-input biosensing, with greater sensitivity as it uses nuclease Csm6 and it is more user-friendly as it implements a lateral flow readout[76].

On the other hand, HOLMES uses CRISPR-Cas12 effectors that carry out cleavage on ssDNA. HOLMES may have advantages in DNA detection, while SHERLOCK is more convenient for RNA detection[77].

Several groups have demonstrated proof-of-concept field deployment systems such as portable colorimetric detection or riboswitch-based methods[78], all of them being closer for out of the lab implementations and exploring easer output readouts to facilitate response interpretation. Some of the examples in this biosensor section is depicted in figure 1.6.

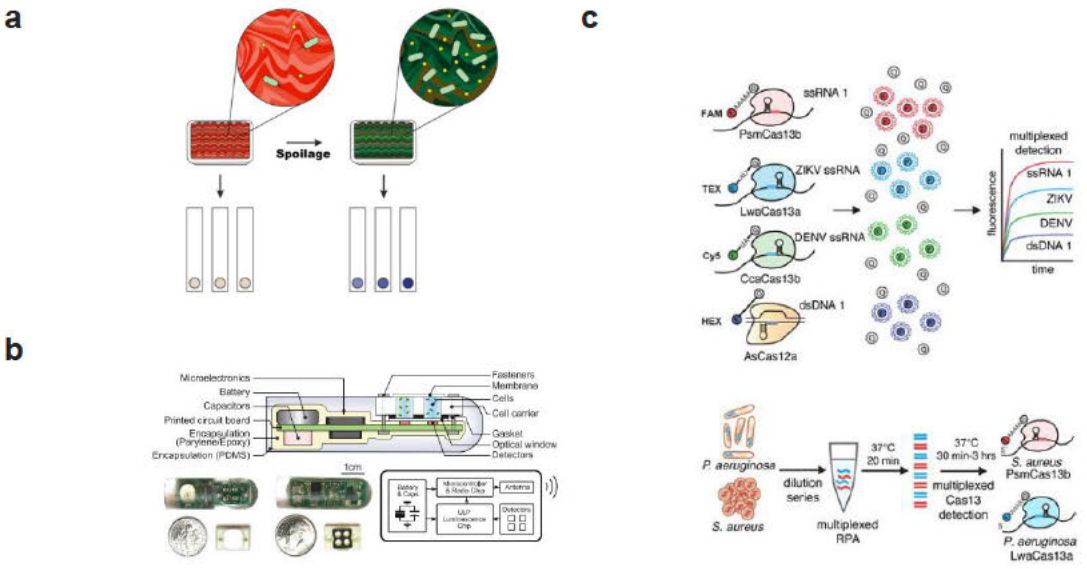


Figure 1.6. Biosensor examples. a. Paper-based whole-cell biosensors for pathogen detection[79]. **b.** Miniaturized wireless sensor with a cross-section of the electrical diagram[70]. **c.** Multiplexed SHERLOCK detection for multiple nucleic acid detection through Cas12 and Cas13[75].

1.3.4 Living therapeutics and probiotic delivery

Therapeutic delivery is a well-documented field, but when being combined with biosensing, enormous options appear for preventive or remote treatment, or even to actuate in places with limited resources and facilities. Ideally, this type of systems

would include a continuous monitoring, a control algorithm and a therapeutic to be delivered[80].

1.3.4.1 Whole-cell closed-loop therapeutics and probiotic delivery platforms

The ability to sense and respond to pathogens has been of great interest. The use of quorum-sensing blocking, i.e., quorum quenching behavior via engineered probiotic organisms, has the potential to combat chronic infection and antimicrobial resistance concerns. For instance, the probiotic *E. coli* Nissle 1917 has been engineered to sense and secrete this type of molecules, as well as an anti-biofilm enzyme to give an extra protection[81]. This probiotic has also been used to sense and responds to nitrate, a biomarker of gut inflammation[82].

Additionally, engineered cellular systems have the potential to sense and treat non-pathogenic diseases such as diabetes and cancer. One recent example involved a closed-loop synthetic gene network in mammalian cells to sense liver disease-associated biomarkers and synthesize a protein therapeutic treatment in response[83]. It utilized cell encapsulation within alginate beads.

Regarding environmental applications, phytopathogens are also studies as alternative methods of chemical pesticides, as these utilize quorum-sensing to regulate the expression of pathogenic phenotypes, thus making quorum quenching strategies could help reduce[84].

1.3.4.2 Biotic/abiotic interfacing

In the case of treatment solutions, orally ingestible cell-based systems are a promising strategy. However, this should be viable over long time periods. Some work has been done on air-drying and lyophilization, but depending on environmental conditions, encapsulation could be a better alternative[52].

Moreover, as these therapies and delivery vehicles are being ingested, biocompatibility tests are required. In the case of implantable devices or stents, it is

important to avoid direct contact of these organisms with the body. In this regard, the use of commensal organisms is important to mitigate any immunological response. Furthermore, additional improvements in targeted drug delivery have been made thanks to magnetotactic bacteria (MTB) that contain iron oxide magnetosomes which enable them to move along geomagnetic field lines via magnetotaxis[85]. For instance, it has been studied that MTB can carry out targeted therapeutic activities such as the killing of *S. aureus* via magnetic hyperthermia upon application of an alternating magnetic field[86]. Continued work in this area can ideally lead to the development of highly functional bio-magnetic interfacing systems for its deployment in outside-the-lab settings. Some of the above-mentioned examples are depicted in figure 1.7.

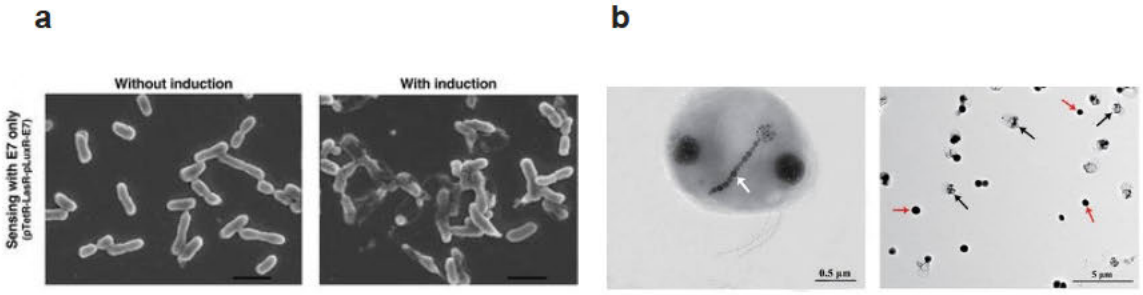


Figure 1.7. Examples for the use of Synthetic Biology in the field of therapeutics. a. On the left, the use of quorum-quenching molecules can be seen, with the effect of the lysis protein damaging cell surface[81]. **b.** On the right, attachment of magnetotactic cells to S. aureus cells can be seen for killing S. aureus cells in vitro and in vivo through magnetic hyperthermia mediated by magnetotactic bacteria [85].

1.4 Thesis Objectives

Living technologies are on its infancy and are proving to become the next focus on the following years to deliver solutions to pressing problems.
The main objective of this PhD thesis has focused on:

Advancing current methodologies applied in the field of Synthetic Biology, i.e., going from more fundamental work to more applied research, to enable more robust, scalable and point of care solutions.
This applied research is fused with deep market analysis to validate and adapt our research to real problems.

Starting with a chapter which meets the computational power embedded in living systems and explores new methodological approaches for synthetic circuit design to break with standard architectures and incorporate space as a new computational element, a series of experimental demonstrations of highly complex computational multi-input logics are implemented. This work demonstrates that the same methodological architecture approach can cover all type of computations, going from digital to analogic, with high scalability potential.

From this first work, a second relevant scenario is explored, deepening into the world of living biosensors. In this next chapter, a whole range of molecules of interest and methodologies are explored, going from chemical compound to biological agents, distinguishing between human-specific pathogens and animal-specific ones. Although different approaches have been taken into consideration, the main objective is to achieve easily deployable solutions for point-of-care diagnostics.

Along this PhD thesis, relevant problems have been tackled, such as heavy metal pollution and bioaccumulation in environmental sources due to anthropogenic activities, the presence of *Vibrio cholerae* in underdeveloped countries, affecting up to 4 million cases worldwide or the presence of pathogenic microorganisms such as

Vibriosis in fish farming, which causes food production losses of up to $5 billion yearly worldwide.

Through this thesis, the following objectives will be covered:

Objective 1. Explore a new methodological approach for robust and scalable synthetic circuit design

- Objective 1.1 Explore the benefits of multicellularity and spatial segregation to achieve greater computational power
- Objective 1.2 Explore the minimal genetics needed to perform complex computations
- Objective 1.3 Explore flexible and tuneable system configurations which would allow digital and analogic behaviours
- Objective 1.4 Define the potential of this methodological approach by defining its automatization, flexibility, scalability, durability, and robustness

Objective 2. Meet the global demand in biosensor's ecosystem by bringing close-to-market solutions

- Objective 2.1 Make use of space to achieve quantifiable sensors capable to sense chemical compounds
- Objective 2.2 Explore the non-triviality of detecting pathogens through their quorum sensing fingerprint
- Objective 2.3 Obtain a pathogen quantification based on the existing correlation between the relative abundances of their quorum sensing fingerprint and the optical density of a certain pathogen after a pre-defined time-window, i.e., pre-culturing time to allow sample growth

- Objective 2.4 Design prototype to create closer to market biosensors solutions

- Objective 2.5 Explore the challenges of bringing Synthetic Biology approaches in underdeveloped countries, aiming to sense human-specific pathogens (SENSA.CHOLERA PROJECT)

1.5 Thesis Outline

Below there is a more detailed explanation of the following chapter which would cover the above-mentioned thesis objectives.

In **Chapter 2**, a more fundamental work is being carried, exploring the potential synergies that can be encountered between printed electronics and synthetic biology circuits. Here, space and circuit element insulation will be clue for circuit design, as well as interconnections with a single wiring molecule, i.e., mimicking electricity from its counterpart. Moreover, different surfaces and scalability approaches are being explored to take the full potential of the proposed methodological approach.

In **Chapter 3**, another big topic is being covered, the art of living biosensing. Although this field has massively been explored, many challenges regarding its commercial availability and point of care deployment are being covered. From these challenges and with a clear technological transference orientation, this chapter explores a way to create quantitative whole-cell biosensors to sense chemical compounds, as well as microbiological agents. To do so, two strategies are explored, being the use of spatial diffusion gradient as a quantification method of certain chemical molecules of interest, and the quantification microbiological agents through a non-trivial quantification of a set of quorum sensing patterns, i.e., diffusible molecules characteristic from bacterial pathogens, and not through the detection of the actual pathogen. Moreover, all these approaches are also studied in commercial terms as I have been participating in entrepreneurial programs to help defining a market need with its further solution.

From this last chapter, i.e., detection of pathogenic agents through its quorum sensing profiles, a spin-off company will be created as the continuity of my professional career. Moreover, it has a patent associated to the methodological process carried on this invention.

CHAPTER 2

2D printed multicellular devices performing computation based on the spatial arrangement

Nowadays, we are witnessing an unprecedented revolution in life sciences technologies, similar to the one experienced in physics and electronics during the past century. Once these living technologies will reach maturity, there will be an impact in our society, complementing, or even replacing some of the current electro-mechanic technologies used in different fields, such as biomedical devices development or industrial applications. In this context, there is a clear consensus that the development of living technologies compulsorily requires the development of complex control systems that integrate multiple inputs and respond according to complex decisions.
In living organisms, cells must sense a wide variety of inputs, both external and internal to survive and reproduce. In response, they compute and actuate several output functions, such as changes in cell morphology, or production of proteins and small molecules[87].

The rise of Synthetic Biology pretends to generate a qualitative leap in our ability to understand, transform and build these complex living organisms into functional synthetic living machines. As it was mentioned before, biological processes imply input and output responses which vary in complexity. For this reason, when considering Synthetic Biology and the construction of these synthetic machines, a new discipline arises, being engineering a key element to fuse both worlds. These synthetic living machines contain genetic circuits, i.e., combination of genes, which responds to inputs and can perform digital binary-like or analogue responses depending on the behaviour exhibited. Most examples of such constructs are proof of concept devices, largely inspired by their electronic counterparts. In this context, it is expected that future designs will become more complex and flexible, most probably following the tracks of the microelectronics revolution.

To date, most synthetic machines, also known as cellular computational devices which use molecular biology parts as the hardware[88], have been created to obtaining useful human-defined behaviours. For instance, the production or delivery of drugs, the environmental monitoring, bioremediation, or biofuel production among others, are some examples described in the literature[89].
Most studies focused on the design of synthetic digital logic gates in living cells. This is made by artificially thresholding biological signals such as chemical molecules, describing signal presence or absence as “0” or “1” (‘OFF’ or ‘ON’, respectively) logic

states. However, there are many challenges when trying to mimic digital logic as biological signals do not generally exist at only two possible concentrations, but usually vary over a wide range of concentrations, being sometimes challenging to set a threshold.

On the other hand, there is the analogue computation, which has received less attention[90]. In this type of computation, inputs and outputs use the range of continuous values between them and calculate mathematical functions on these signals using the laws that govern biochemical phenomena.

After contextualizing the two main natures of computational devices, i.e., digital or analogue, an introduction to how these are implemented in living cells should be made. Over the years, most computational systems have been designed according to a standard architecture based on the abstract model defined by Turing in the 1930s, and the von Neumann architecture used to implement computations performed by the Turing Machine. This architecture organizes their components into three different layers: the input layer, where external signals are detected; the processing layer, where computation is performed; and the output layer, where the final output is produced[91,92,93,94]. Circuits usually respond to biological signals which are thresholded into '0s' and '1s' respectively[95]. Figure 2.1 depicts the theoretical computational model based on those three levels. The downside of this theoretical designs is its further experimental implementation as for instance, no such a sharp "ON" and "OFF" states exist in nature, making difficult to define Boolean logics[96], as well as the output response variability and system scalability.

Despite there have been many advances on the use of simulation tools to guide circuit design inspired on electronic design automation (EDA)[97,98] (i.e. a process that consists of selecting the appropriate components and genetically modifying existing components until the desired behaviour is achieved) or perform directed evolution to optimize genetic circuit behaviour, issues regarding scalability, reusability, engineering complexity and technological transference remain a challenge.

Below, figure 2.1 displays the basic elements involved in a computational design and how biology and computation can be fused.

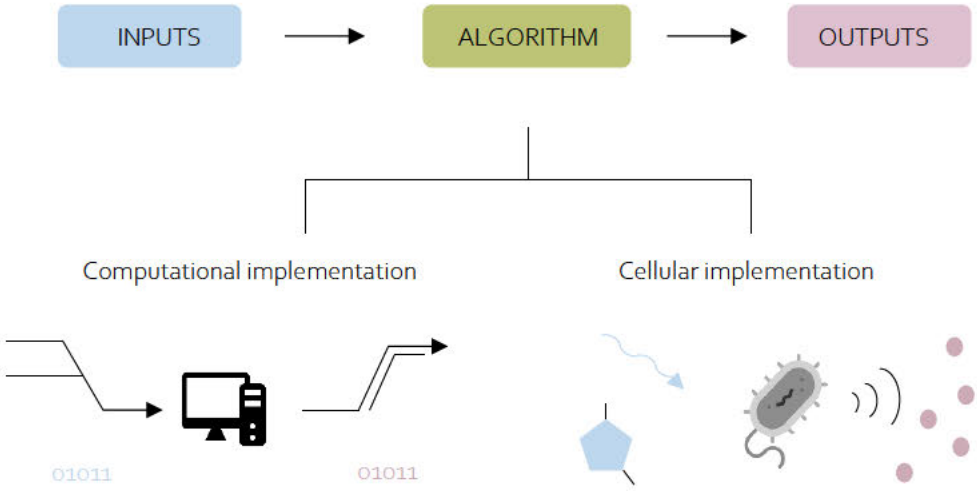


Figure 2.1. Basic computational design elements. This image depicts the main fundamental layers of computation, i.e., input, processing, and output layers. Furthermore, the analogy between engineering and biology is also depicted, as both systems need input signals to undertake a certain computation that will lead to an output response.

Although a lot of research pointing towards this field has been made, there are still many challenges to be overcome.

The overemphasis on digital designs has offered powerful success over the past few decades. The way to obtain more complex computations is by assembling simple digital logic units which, regardless of electronics, are wired based on molecular interactions. Thus, instead of having isolated components which are physically interconnected by a wire, highly orthogonal genetic parts must be obtained, making difficult their scalability.

This inefficiency is translated into metabolic or toxicity burdens on cells which have a limited number of resources. When comparing to electronics, there are five to six orders of magnitude fewer genes per cell than digital transistors per chip, meaning that the use of genes to only perform digital computation is not a great way to achieve

complex designs[99,100]. Additionally, because of the intrinsic difficulties of implementing them, the resulting constructs are usually specific for the given problem and cannot be reused afterwards.

On the other hand, analogue computation is useful for signal processing when the output needs to be dependent on graded information or continuous functions of the inputs[101]. Thus, it might seem easier to perform complex computations as component complexity is simplified by the underlying physics of the components. Moreover, analogue computation is an advantageous alternative to digital logic in terms of efficiency regarding space, time, or energy[27,102]. Nevertheless, analogue signal integration is susceptible to noise, making it challenging to design robust synthetic genetic architectures[103] and this type of devices are just starting to be understood, so a lot of research needs to be done[104].

To solve some of the above-mentioned challenges, nonstandard approaches have already been explored, such as using cell consortia to distribute the output signal among different cell types. The use of multiple cells for computation is known as “distributed computation”. The ability of separating molecular components within several engineered cell types is appealing as cells can be combined in multiple, reusable, and scalable ways[105].

Living systems harness the capabilities of multicellular ensembles, so when distributing computational demands across a population of engineered cells, the burden on each individual cell is lowered down, offering a way to easily scale the complexity of the computations being performed[106].

These systems work dynamically thanks to mechanisms of cell-to-cell communication patterns. In these patterns a given cell gathers signals and sends an output that is received and properly processed by a receiver cell. This configuration is optimal to physically separate different molecular components.

When allocating different tasks to different cells, it is possible to simplify the required computation at different levels. Cellular separation enables circuit elements reusability thanks to the physical insulation of intracellular components, facilitates circuit

implementation as the elements introduced in each cell are reduced and lowers noise due to the mixture of the wiring molecules present in the media from each sender cell, and hence the receptor cells sense the wiring molecule from different cells. Only when the signal is strong enough (from different cells) a response will occur. Thus, spurious, or noisy responses will be abolished. Spatial segregation and the use of cellular consortia offer the appropriate conditions for achieving complex designs[107]. Figure 2.2 displays this concept of spatial segregation and distributed computation in biological systems.

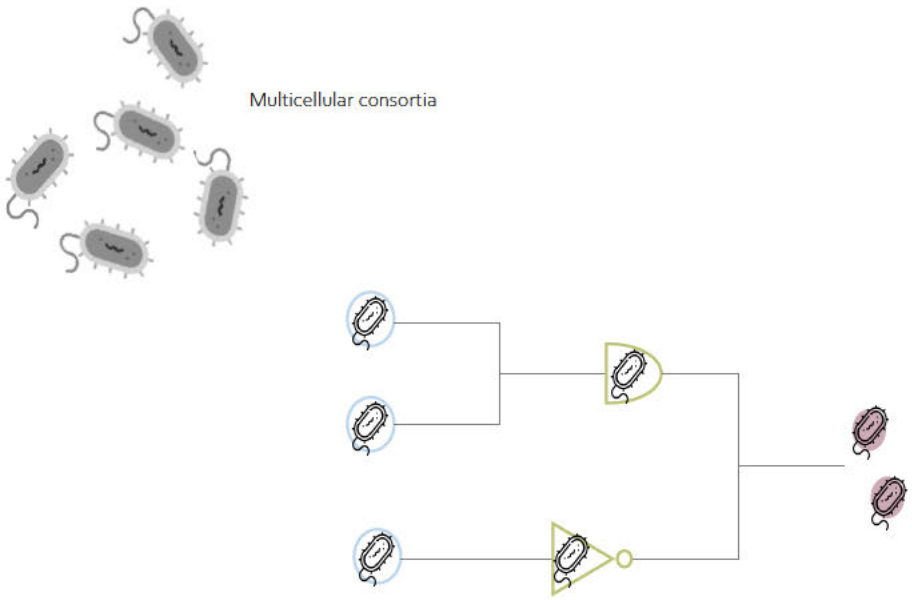


Figure 2.2 Theoretical design of a genetic circuit making use of distributed computation. This image shows a scheme where each living cell performs a certain aspect of the computation, having this distribution concept to perform complex computations thanks to cellular consortia.

OBJECTIVES

The main goal of this chapter it to create a new methodology to design simple, scalable, robust, and cost-effective multicellular devices to develop both digital and analogue circuits. For it, we have taken inspiration from printed electronics, introducing the space as a new computational element, so the computational complexity is encoded on the circuit topology and not in the genetics.

By encoding circuit configurations in a certain spatial pattern, we believe it will be possible to implement any given truth table, using a short library of engineered cells. With this approach, we believe that elements will be able to be reused and we will obtain robust circuits with a huge potentiality to be scaled at industrial level.

This proof-of-concept can represent a breakthrough scientific and technological towards the development of future living technologies in the long-term.

This chapter is organized in several sections to achieve the following specific objectives.

1. Methodological setup for robust and scalable circuit design, using 2D surfaces as a new computational element
2. Approach validation by mapping any arbitrary truth table to a pre-defined multi-branch topology, where each branch behaves as a multi-base transistor
3. Achieve complex computations ranging from digital to analogic through experimental validation
4. Setup automatization and features description such as durability, stability, and scalability

2.1 Basis of the new methodological framework and theoretical definition of a minimal functional complete set

2.1.1 New methodological framework

A cell-based logic network consists of engineered cells which, upon a certain input pattern, produce an output macromolecule. The mechanism to dictate the computational rules, is commonly based on the use of transcriptional regulators, transcription factors, polymerases, receptors, or recombinases[108,41]

During last decade, important efforts have been devoted to developing cellular computational devices using different strategies. However, the hard truth is that nowadays there is not a well-established technology that allows the creation of computational devices in a standard, reliable and scalable way. In consequence, current state-of-the-art makes not possible the technological transference to end-user applications.

The main reason for this technological limitation relies in the lack of new methodologies for circuits design. In general, researchers tried to apply the same principles for cellular circuits that used in electronics. Therefore, enormous efforts have been devoted to creating logic gates or other basic components to obtain simple functional units. Then, by just connecting these elements properly, any arbitrary circuit would be built. This analogy is theoretically straight forward. Conversely, many problems arise when trying to implement it such as gene expression variability, mutation, cell death, genetic complexity, metabolic load, changes in the extracellular environments and improper interactions with the cellular context, among others.

Moreover, the difficulty of connecting logic gates in a cellular environment, the effects of the metabolic burden or the enormous variability present in living systems are also very important limiting factors.

In summary, biological components have some specific features that make the creation of computational devices more difficult.

We believe that the advance of living technologies demands a completely new approximation to overcome the above-mentioned limitations. In this project we propose the development of a new approach for cellular circuits design, discarding previous methodologies and recovering the basic fundaments of computation and taking advantage from multicellular embodiment.

For it, we have explored a new framework for computational devices based on biological substrates completely different from the standard architecture, as no distinctions between input and processing layers are considered. By breaking with the conventional architecture and introducing the space[109] as an additional computational element[110,105], we have demonstrated that simple, robust and scalable multicellular computational devices can be built. This methodology is inspired from printed electronics[111,112,113,114,115], and it proposes the creation of circuit pattern, using multicellular architectures to accomplish the computational requests. Printing technologies in electronics have allowed the current production of circuits at industrial level as it ensures reproducibility and scalability at low cost.

Considering that computation is, in essence, a matter of information processing[116], our approach encodes the computational information in the concentration of a unique biological signal, the carrying signal (CS)[117,118].

This CS, which is produced at a specific location in the device, can diffuse across the 2D space. While diffusing, the CS can interact with the different device components which are distributed on the surface in a specific spatial arrangement. The main role of these components is to modulate the flow of the CS in one of two different ways: these can either allow, or even amplify the flow of the CS across them or can reduce or abolish the CS flow. When these modulatory elements act by only allowing or blocking the CS flow, the device performs digital computations. On the other hand, when regulatory elements increase or reduce the concentration of the CS, and these variations are meaningful in terms of output production, the device performs analogic computations. Figure 2.3 represents this new architecture which breaks with conventional logic gates architecture and uses computational active 2D surfaces to perform computation based on specific spatial arrangements.

The ability of codifying the computational complexity in the topology of modulatory elements, i.e., in their specific spatial arrangement, enables the construction of systems that have very low genetic complexity but that are capable of implementing complex computations[119]. It should be noted that in these devices, the spatial sequence in which the CS meets the different modulatory elements defines the computation. Thus, the same modulatory elements can implement different computations by just altering their spatial position.

The use of a multicellular implementation for computational devices has been shown to be very useful for complex circuits[109,120,30,121,] as each component is implemented in a different cell type. Moreover, this embodiment is appealing because it simplifies the genetic engineering required, thereby minimizing the emergence of unexpected interactions with the host cell and significantly reducing the metabolic burden associated with foreign genes[122,123]. Furthermore, the growth of different cell types in different spatial locations provides devices with high stability since negative competition effects[124,125], which emerge when multiple cell types grow in the same media are avoided.

Additionally, stamping living multicellular circuits on a solid surface is appealing because it offers the possibility of using commercial printers to create all type of devices. This will provide an easy method for automate devices production in a reduced cost. Moreover, circuits printed on solid substrates will be easier to store and manage

Hence, the first requirement for this new methodology is that cell types composing the computational device should be able to be deposited on the surface in the right position and the carrier signal should diffuse along this surface connecting all these different circuit units. For it, the first surface to be tested would be paper due to its versatility, low-cost and scalability advantages.

Combined with the circuit topology requirements, it is necessary to define the minimal set of modulatory elements that define a functional complete set, i.e., any arbitrary device can be implemented combining only these elements. In our approach, each one of these elements will be implemented in a different cell type that, depending on

external inputs, will perform different modulations of the carrier signal. The following figure (Fig.2.3) displays the elements involved in signal processing and the role the 2D surface plays to achieve the desired computational behaviour.

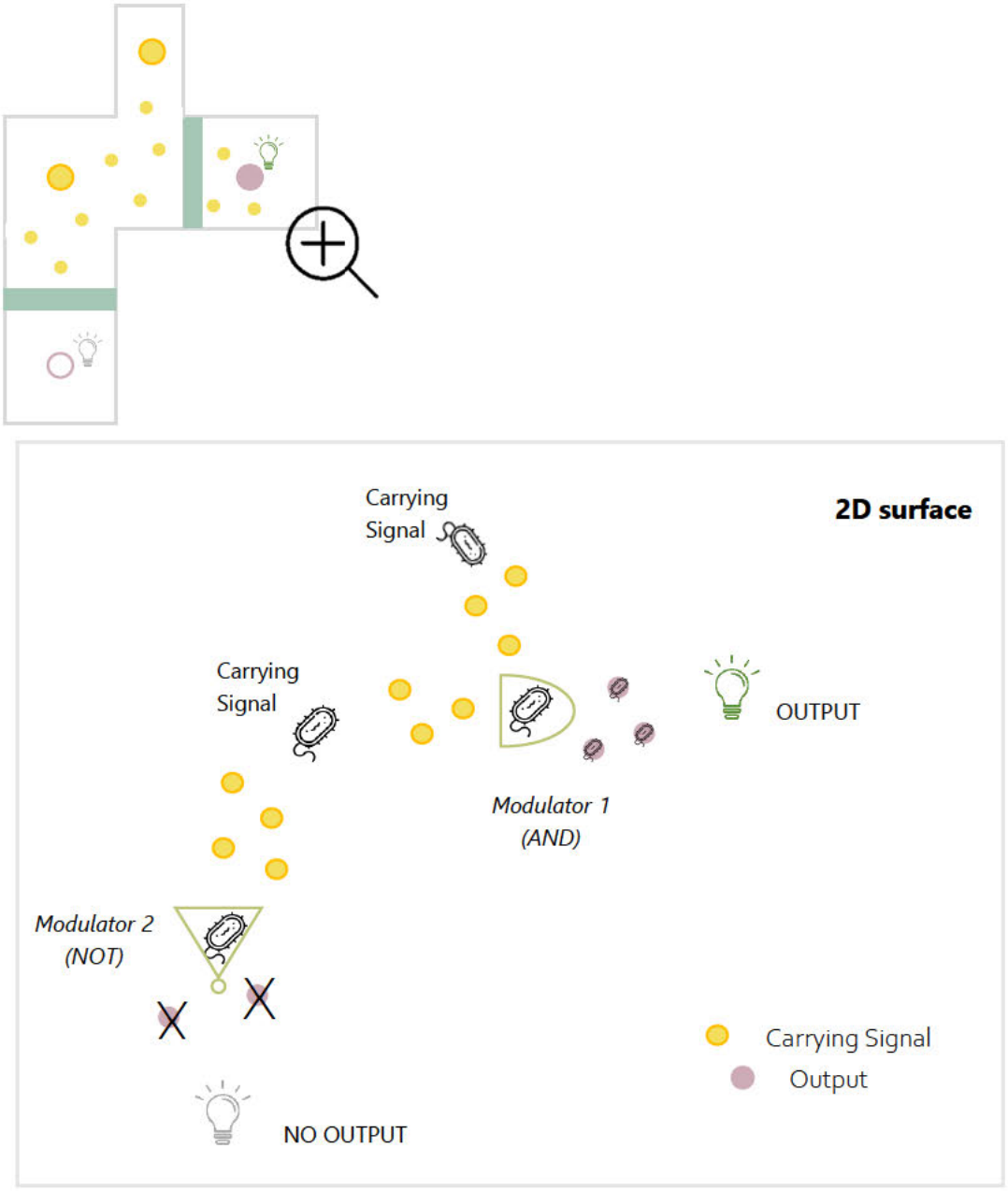


Figure 2.3 2D surface with computational capabilities. This image depicts the logic behind the methodological approach proposed in this study. The idea is to have a computationally active surface thanks to our CS, which contains the signal information. Then, to achieve a specific computational behaviour, different modulatory elements will be displayed in a specific spatial arrangement to modulate this CS flow. In the example, there are two modulatory elements. One follows an AND logic and a second one performs a NOT logic. These modulatory elements are specifically designed to allow or not the flow of the CS, which will ultimately dictate whether an output is present or not.

2.2 Construction of a library of genetically engineered cells

2.2.1 Genetic Engineering

As mentioned in the first objective, the main elements needed to construct our circuits are: i) CS supplier cells, to generate the signal, ii) Modulator cells to regulate this signal, iii) Reporter cells to produce the final output.

First, three cell types, S_1, S_2, S_3 were engineered to produce constitutively (S_1), or upon external induction (S_2 upon arabinose and S_3 upon rhamnose), a small extracellular molecule that acts as the CS. This molecule is the bacterial 3OC6HSL acyl homoserine lactone (AHL) which is involved in the natural quorum sensing mechanism[126,127]. AHL molecules can diffuse along surfaces and thereby encounter the different modulatory elements. In our design, unlike in standard methods, there are no explicit connections between the different components of the circuit. Instead, each component contributes independently to the final computation by partially modifying the AHL flow.

Regarding the modulatory elements, these elements were implemented in different engineered cell types that express, or do not express Aiia, an enzyme that degrades AHL,[128,129] in response to external inputs spread over the whole device surface. More specifically, some modulators (M- cells) reduce or even abolish the flow of AHL by inducing Aiia expression upon the presence of an external inducer**.** In contrast, in other modulators (M+ cells), Aiia is constitutively expressed, and external inducers block this constitutive expression, thereby increasing the flow of AHL. Finally, to compensate the AHL concentration decrease associated with the diffusion process, an auto-amplifier element (CA) was also created. This auto-amplifier cell can produce AHL only when AHL is detected, and in this way, restores the AHL signal.

The final overall circuit response is embodied in a specific cell type (reporter cell, CR) that produces the output only when the CS concentration exceeds a given threshold. As a proof-of-principle we expressed a green fluorescence reporter protein (GFP) as the final output.

If not specified, all cells were constructed using E. coli Top10 (DH5-alpha) strains from Invitrogen which constitutively synthesizes tetR. For some inducible constructs, Zn1 strain, where tetR protein is constrictively expressed allowing the control of Ptet promoter by using Anhydrotetracycline (aTc) as an induced

i) **CS supplier cells**: These cells produce AHL molecules, acting as CS, in different locations of the circuit surface.
   - For digital circuits, *cell S1* was built to produce AHL molecules in a constitutive manner by expressing LuxI downstream P_{Tet} promoter.
   - For analogue circuits, *cell S2* was built, allowing the production of AHL in response to an external input, i.e., arabinose, expressing LuxI, synthase, responsible of AHL synthesis[127], downstream the arabinose-inducible promoter P_{ara}
   - Additional inducible supplier defined as *S3*, producing AHL molecules upon the presence of other external input, i.e., rhamnose $P_{rham.}$

ii) **Modulator cells**: These cells are responsible to modulate CS, expressing Aiia an intracellular enzyme which catalyses the degradation of AHL signal. Here there are two subsets of cells.
   - Negative modulators reduce the AHL concentration in the presence of external inputs. This is done expressing the Aiia enzyme downstream an externally inducible promoter. Specifically, M^-_{ara} expresses Aiia under the regulation of the arabinose-inducible promoter P_{ara}[130], whereas M^-_{aTc} expresses aiiA upon P_{Tet} promoter[131], which is constitutively repressed by TetR in Zn1 strain. In the presence of Anhydrotetracycline (aTc) P_{Tet} promoter is depressed and aiiA is expressed. Thus, we used a different E coli strain for this modulator.
   - Positive Modulators allow the AHL diffusion. These cells express LacI repressor under externally inducible promoters, P_{ara} (M^+_{ara}) and P_{Tet} (M^+_{aTc}). Finally, the P_{Lac} promoter [132], which is repressed by LacI, regulates aiiA production. This means that upon the presence of an external input, LacI is expressed, inhibiting P_{Lac} promoter to transcribe the Aiia enzyme. Then, AHL diffusion is allowed.

iii) **Amplifier cell (CA):** To compensate AHL decay due to diffusion gradient, this cell produces AHL in the presence of AHL. The implemented architecture is based in the Lux system from *vibrio fischeri*[133]. Receptor protein LuxR, which is constitutively expressed, binds to AHL molecules forming an active dimeric complex. This complex triggers the expression of LuxI under the P_{Lux} promoter

iv) **Reporter cell (CR):** To validate the correct behaviour of the cellular circuits, these cells produce GFP in response to AHL levels. This cellular architecture is based on the same Lux system than CA cells, but in this case, these express GFP under P_{Lux} promoter, instead of LuxI. These last cells produce the circuit final output.

To experimentally validate our approach, we built this library of engineered cells. The genetic architecture of each cell type is schematically shown in figure 2.4 and each specific genetic part is summarized in ANNEX 2 (Table S3.1 and S3.2).

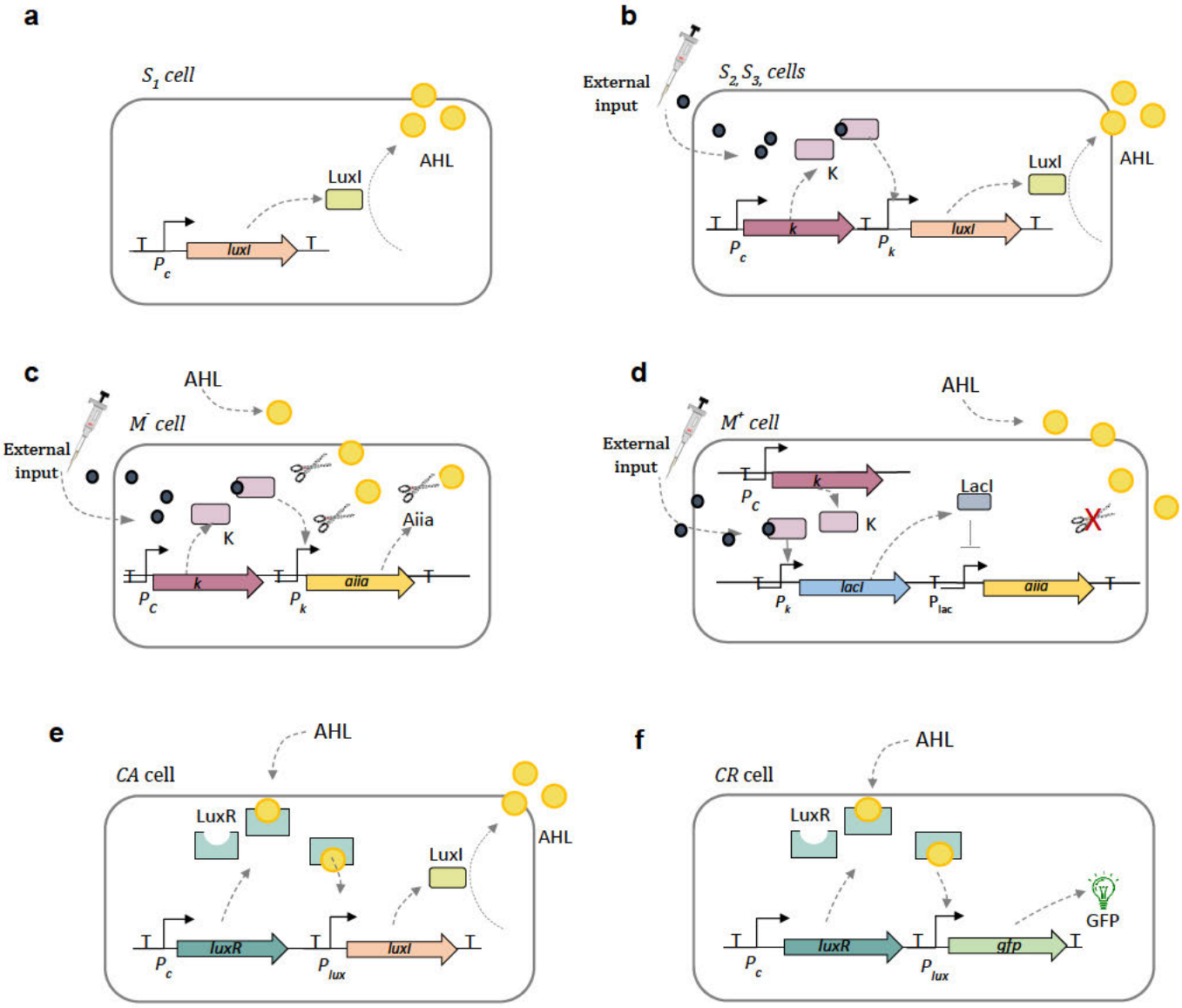


Figure 2.4. Genetic architecture of the cell library. a. S_1 cells express LuxI downstream of the constitutive promoter P_C. LuxI generates AHL molecules which are secreted. **b.** In S_2, S_3 and S_4 cells, the expression of LuxI is regulated by an externally inducible promoter P_k (arabinose-inducible promoter P_{BAD} in S_2 cells and rhamnose-inducible promoter P_{rham}. K represents the receptor protein, AraC, RhaR and MerR respectively. **c.** In negative modulatory cells M^-, Aiia expression is regulated by an external inducible promoter P_k (P_{BAD}, Ptet and Pram respectively). The corresponding receptor proteins K are Arac, aTc and RhaS respectively. **d.** In positive modulatory cells M^+, the expression of LacI repressor is regulated by an external inducible promoter P_k (similar to negative modulatory cells) which in turns, negatively regulate Aiia expression. For b, c, and d, the external input (green circles) is either aTc, arabinose or rhamnose. **e.** Auto-amplifier cells (CA) express LuxI in the presence of AHL, which binds to and dimerises the LuxR transcription factor, that subsequently induces more AHL. **f.** Reporter cells (CR) produce GFP as a final output in the presence of AHL. Symbol T represent a double-terminator sequence.

2.2.2 CR construct characterization

To start characterizing the different computational units for circuit design, the relationship between our carrying signal (CS) concentration and reporter cells (CR) response through GFP expression was studied. This correlation is displayed in Figure 2.5, where CR cells response were analysed upon the addition of different synthetic 3OC6HSL (AHL) concentrations, i.e., from 10^{-4} to 10^{-9} M for an overnight at 37°C. In this case, we carried experiments in liquid cultures to assess system response from our receivers.

In Figure 2.5, experimental results are represented with dots, which are fitted to a mathematical function represented by the solid line.

$$GFP\ abs = \alpha_{AHL} + \frac{k_d \cdot [AHL]^n}{k_{max} + [AHL]^n} - \delta_{GFP}[GFP] \quad (1)$$

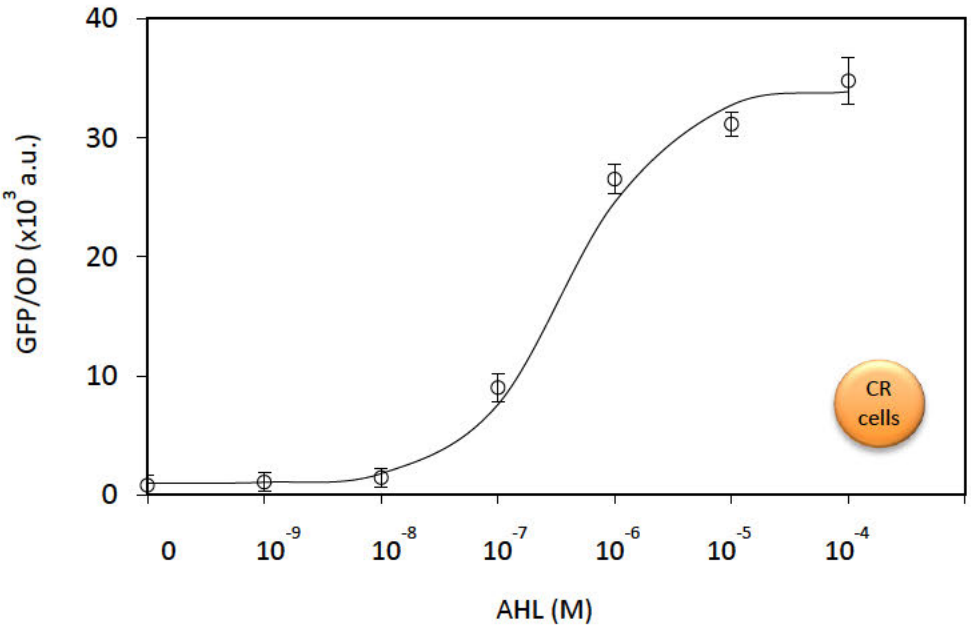
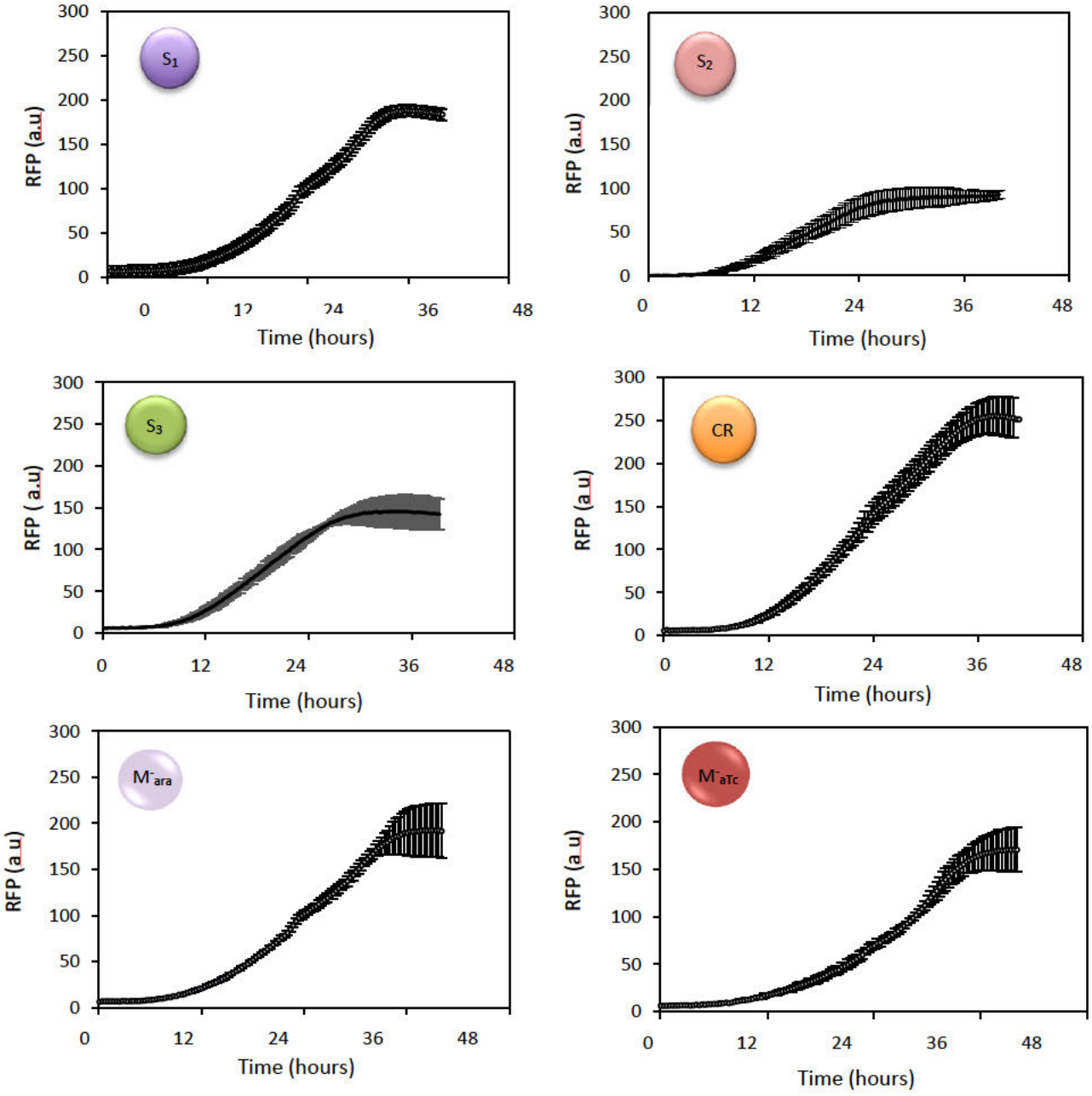


Figure 2.5. AHL Transfer function of CR cells. Dots are the normalized GFP expression of CR cells grown liquid cultures at different AHL concentrations. The solid line indicates the mathematical data fitting, according to equation (1). Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

2.3.2 The use of RFP to assess cell density

When performing our circuit measurements, we observed that working surface, i.e., in this case, paper strips as a first approach, absorbance was too high and cell density was impossible to be measured. For this reason, we used constitutive levels of RFP as an indirect measure of cell density. Figure 2.6 shows the temporal evolution of RFP expression associated with cell growth in the various cell types.



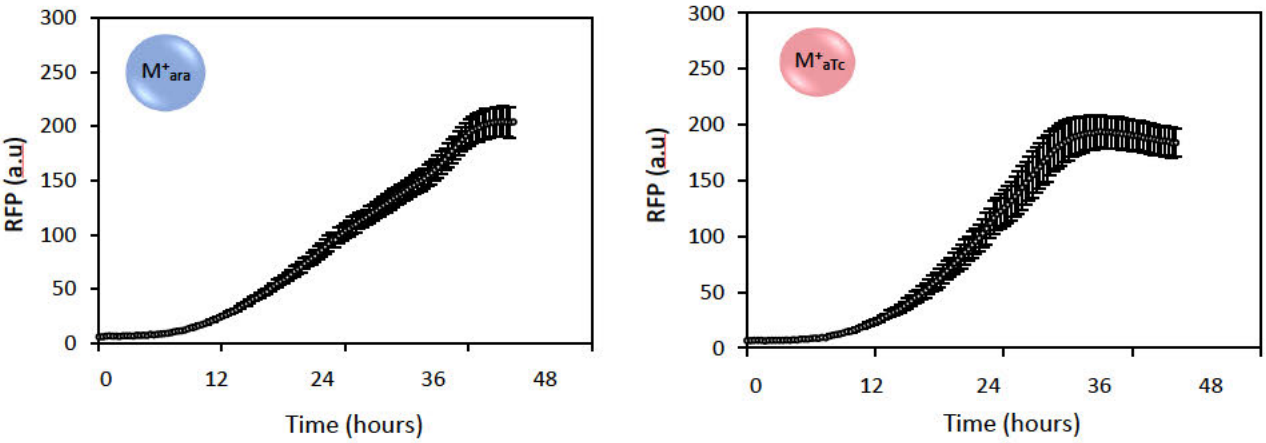


Figure 2.6. Cell growth based on constitutive RFP expression. Increase in red fluorescent protein (RFP) levels over time associated with cell growth in each indicated cell types. Error bars are the standard deviation (SD) from three independent measurements. Data are presented as mean values +/- SD.

Experimental results indicate similar dynamic growths for the different cell types, considering RFP a good indirect indicator of cell growth.

2.3. Creation of cellular inks for cellular spatial patterns

To take full advantage of our approach, we also developed a set of cellular inks composed of a single cell type mixed with cellular nutrients and agar (used as a thickener). These inks were used to draw the different circuits in the paper.

Cellular inks were elaborated by preparing a cell culture from single colonies obtained from streaked glycerol stocks and grown until reaching an optical density (OD) of 0.2. From these cultures, two different cellular inks were elaborated, one for ready-to-use circuits and another one to have circuits stored at -80 ºC. For the first ones, these cell cultures were mixed with LB agar, prepared by adding 0.6 g of agar to 50 mL of LB and boiling it until having everything diluted. After cooling 50 ºC, properly antibiotic selection and cell cultures were added to obtain each cellular ink. The use of agar in the mixture prevents cell culture dispersion when deposited on paper surface,

providing some cellular confinement and to have a better control of the positioning of each cellular element.
More specifically, for the modulatory elements, a volume of 100µL of cell culture was mixed with 50µL of liquid LB-agar mixture. For the rest of elements, i.e., the S_1, S_2, CA and CR cells, a volume of 50µL of cell culture was mixed with 50µL of LB-agar, also in liquid state.

2.4. Circuit substrates: how to explore the space using different topologies

Despite there are several suitable materials that could act as the working substrate, paper was selected due to its low cost and its ability to allow bacterial growth and AHL diffusion. It is worth mentioning that previous studies have also used paper for different cell-based applications or organic transistors due to these optimal properties[134,135,136].

To endorse the proposed methodology, we first analysed cell deposition and cell growth on a paper substrate, as well as CS transmission along the surface. For it, the CR cells have been characterized in a solid surface. A 96-well plate was filled with known dilutions of AHL mixed with LB agar, from 10^{-4} M to 10^{-10}M. After, small pieces of paper were placed on the top of each well and a dot of 1µL of reporter cells (CR) was added.

Moreover, each of the experimental data sets was mathematically fitted to a sigmoidal Hill function and parameters can be found below (Table 2.1).

Results are displayed in Figure 2.7, comparing results from liquid cultures (Figure 2.5) and results using a solid surface.

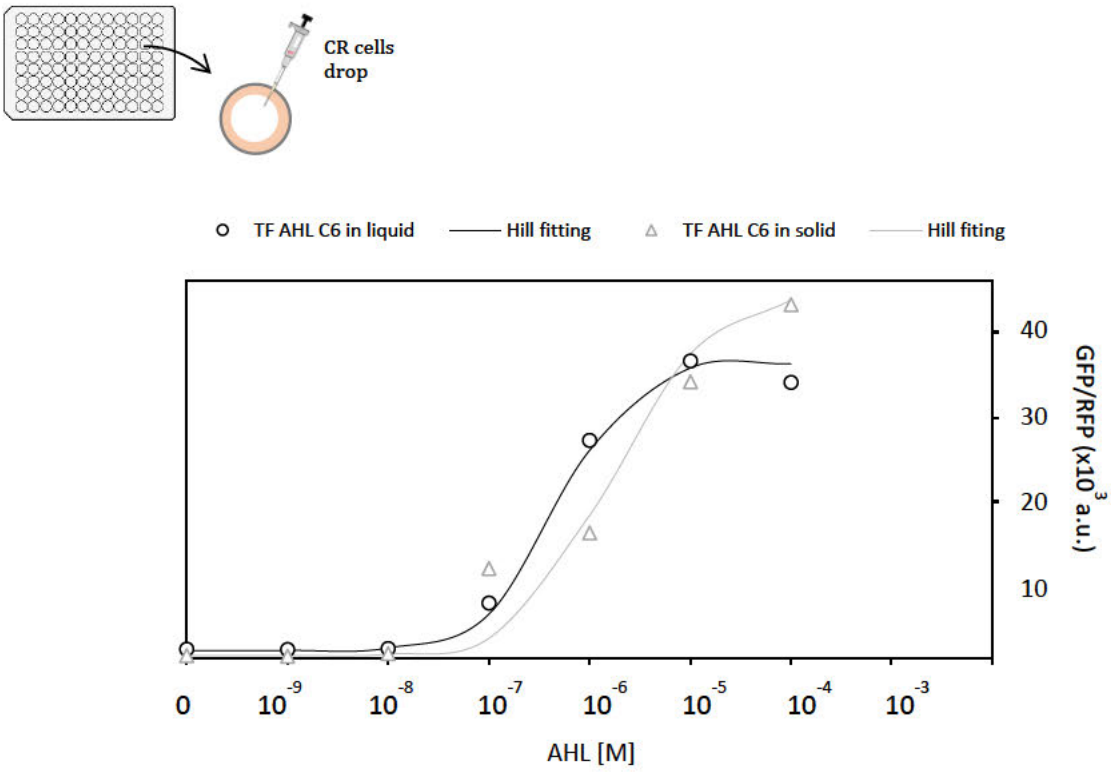


Figure 2.7. AHL C6 transfer functions. The chart above represents the activation of the reporter cell in different induction ranges (from 10^{-4}M to 10^{-10}M). The experimental data in liquid and on agar plates is represented in black dots and grey triangles respectively, each of them with its corresponding mathematical fitting to a Hill function.

Parameters	Values	
	In liquid	In solid
α_{AHL}	400	117
k_d	5: 10^{-8} μM · min^{-1}	1.5·10^{-7} μM · min^{-1}
k_{max}	1.6·10^4 μM · min^{-1}	2·10^4 μM · min^{-1}
n^{hill}	1.2 a.u	1.1 a.u
δ_{GFP}	1 min^{-1}	1 min^{-1}

Table 2.1. Fitting parameters. This table displays the parameters used for the curve fitting for AHL transfer function in Fig.2.5 and 2.7.

Next, the diffusivity was studied. In this process, distances are a crucial point to build a perfect connection between elements. From previous literature[137–139], it is known that these small AHL molecules can diffuse in all directions and can pass across cell membranes.

Figure 2.8a shows a spatial pattern composed of a constitutive AHL supply (S_1) at one end of the strip and reporter cells (CR) uniformly distributed along the rest of the surface. Quantification of the GFP produced in the reporter cells indicates that S_1 cells can grow in the surface and secrete AHL, which efficiently diffuses along the paper and can be detected by the CR cells at large distances, i.e., up to 20 mm away from the AHL cell source.

The ability of AHL auto-amplification by CA cells, located between S_1 and CR cells, to restore the signal decay due to diffusion is also analysed in figure 2.8b. Experimental results demonstrate that CA cells can extend the range of the diffusing CS.

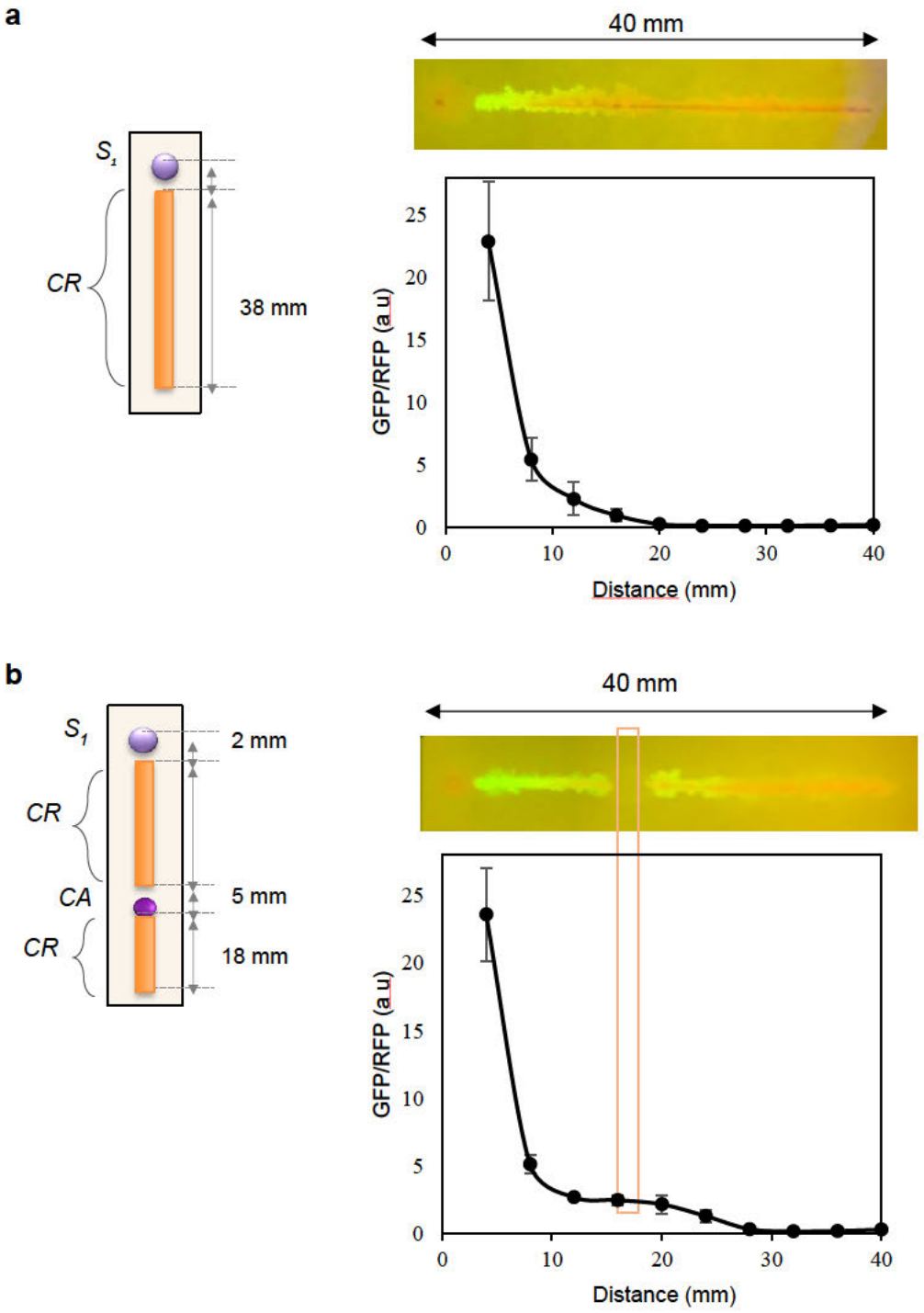


Figure 2.8. AHL signalling through paper diffusion. a, b, Left. Schematic representation of the different cells used at each of the indicated distance points along the paper. S_1 cells, constitutively produce AHL; CR cells are GFP reporter cells; CA cells are auto-amplifier cells inserted to restore the signalling. **a, b,** Right. Numerical quantification of the GFP levels along each strip, acquired by scanning the surface of the paper after incubation during 24h at 37ºC. GFP levels are normalized by RFP, which correlates with cell population. Error bars are standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

2.5. Definition and experimental validation of 2D patterns implementing different multicellular circuits

2.5.1 Transistor-like circuits

Once seen that cells can perfectly grow and diffuse signals through paper substrates, we analysed the effect of CS modulation on the final output by locating different modulatory elements between S_1 and CR to implement more complex devices. The resulting circuits can be compared to electronic transistor architecture, in which the CS, i.e. AHL molecules, enter into the device (source), are modulated in response to an external signal (gate) and then exit the device (drain)[140]. Figure 2.9 shows an example of a transistor-like circuit where all components can be directly mapped onto our circuit topology. Figure 2.9b shows the behaviour of the device. In the absence of the external input, i.e., arabinose, the CS (AHL molecules) is constitutively produced by S_1 cells and diffuses along the surface, activating GFP production by the CR reporter cells. In the presence of arabinose (10^{-3}M) the modulatory element M_{ara}^{-}, inserted between the S1 and CR cells, produces Aiia, which degrades AHL, thereby preventing further AHL flow.

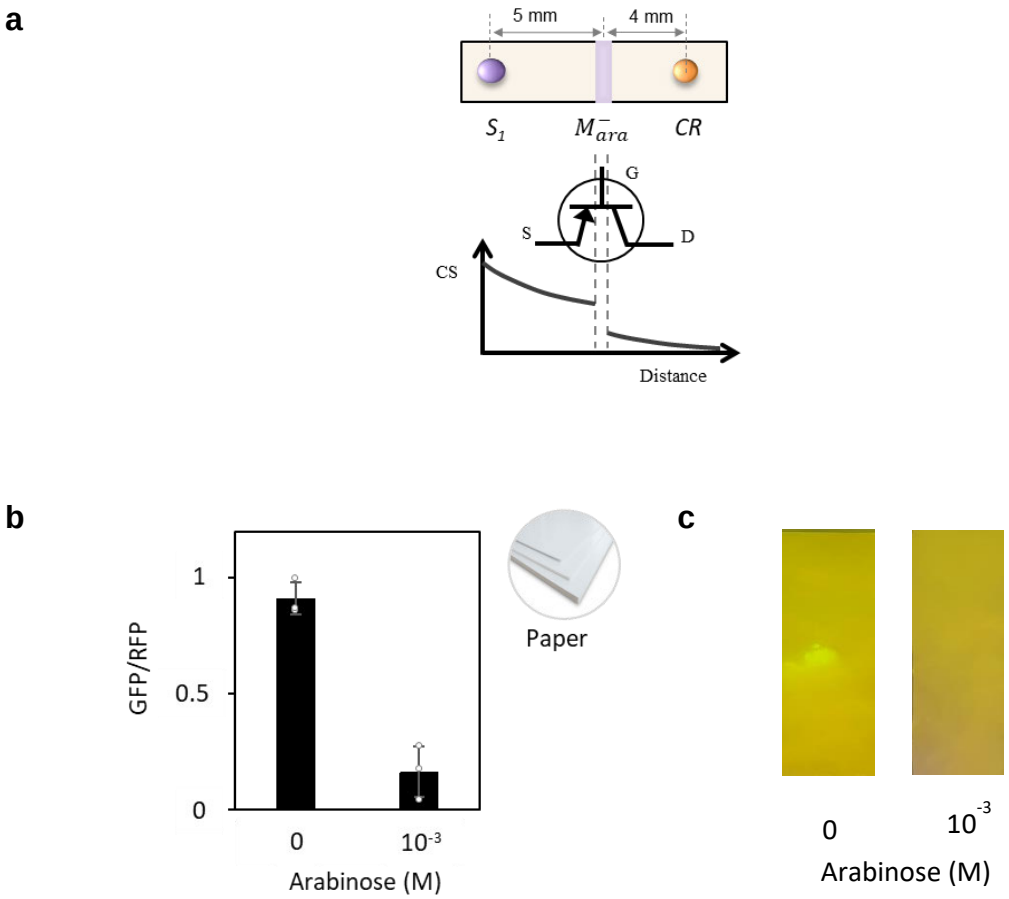


Figure 2.9. Printed circuit implementing a transistor-like device. a. Mapping of a general transistor architecture on a cellular printed pattern. Similar to the transistor architecture, the cellular pattern is composed of three main components: source (S_1 cells), gate (M^- cells) that responds to external inputs and a drain (CR cells) as the final output responding to the presence of the carrying signal (CS). **b.** Circuit response. In the absence of external input, i.e., arabinose, the CS encoded in the production of AHL molecules by S_1 cells diffuses along the surface, inducing GFP expression in reporter cells CR. In the presence of 10^{-3} M arabinose (Ara), the modulatory element M^-_{ara} produces the AHL cleaving enzyme Aiia, which degrades the CS. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. Experiments are performed on paper strips. The average fold change is 5.6x. **c.** Photography of the device.

2.5.2 Modulatory elements

From this first transistor-like architecture, we analysed the CS modulation. Specifically, six different modulatory elements were tested, each of them responding to one of two external signals, namely aTc, arabinose and rhamnose. Three of the modulatory elements (M^{+}_{aTc}, M^{+}_{ara} and M^{+}_{rham} cells) allows the CS flow across the circuit when the level of the external signal increases, whereas the other three modulatory elements (M^{-}_{aTc}, M^{-}_{ara} and M^{-}_{rham} cells) reduce the CS flow when the level of the external signal decreases. The theoretical behaviour of these modulatory elements is represented in figure 2.10.

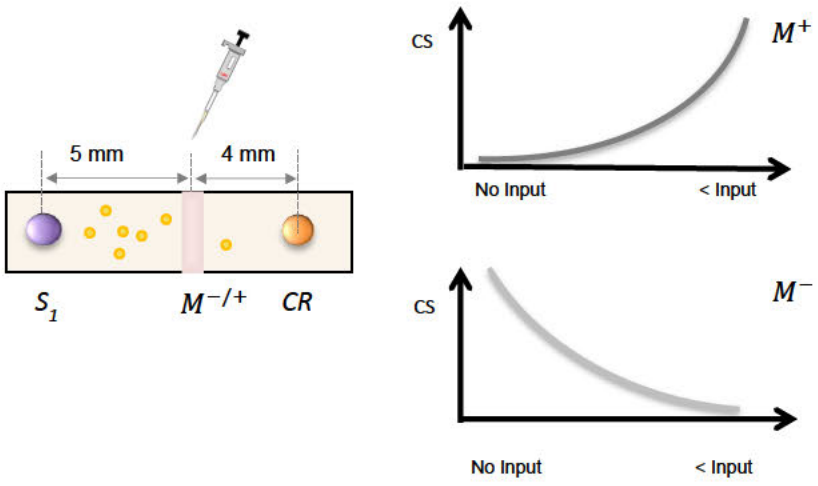


Figure 2.10. Analogic signal modulation through external input. This image represents the two different modulators used in our approach. Those can be modulated with different concentrations of external inputs, obtaining the behaviours depicted on the right.

The experimental validation is shown in figure 2.11, demonstrate that these modulatory elements are, in essence, analogue, allowing the tuning of CS levels in a continuous manner. This figure displays the output data relative to cell population, i.e., ratio of GFP/RFP. In consequence, by properly combining these modulatory elements which respond to specific external inputs, either analogue or digital circuits can be implemented.

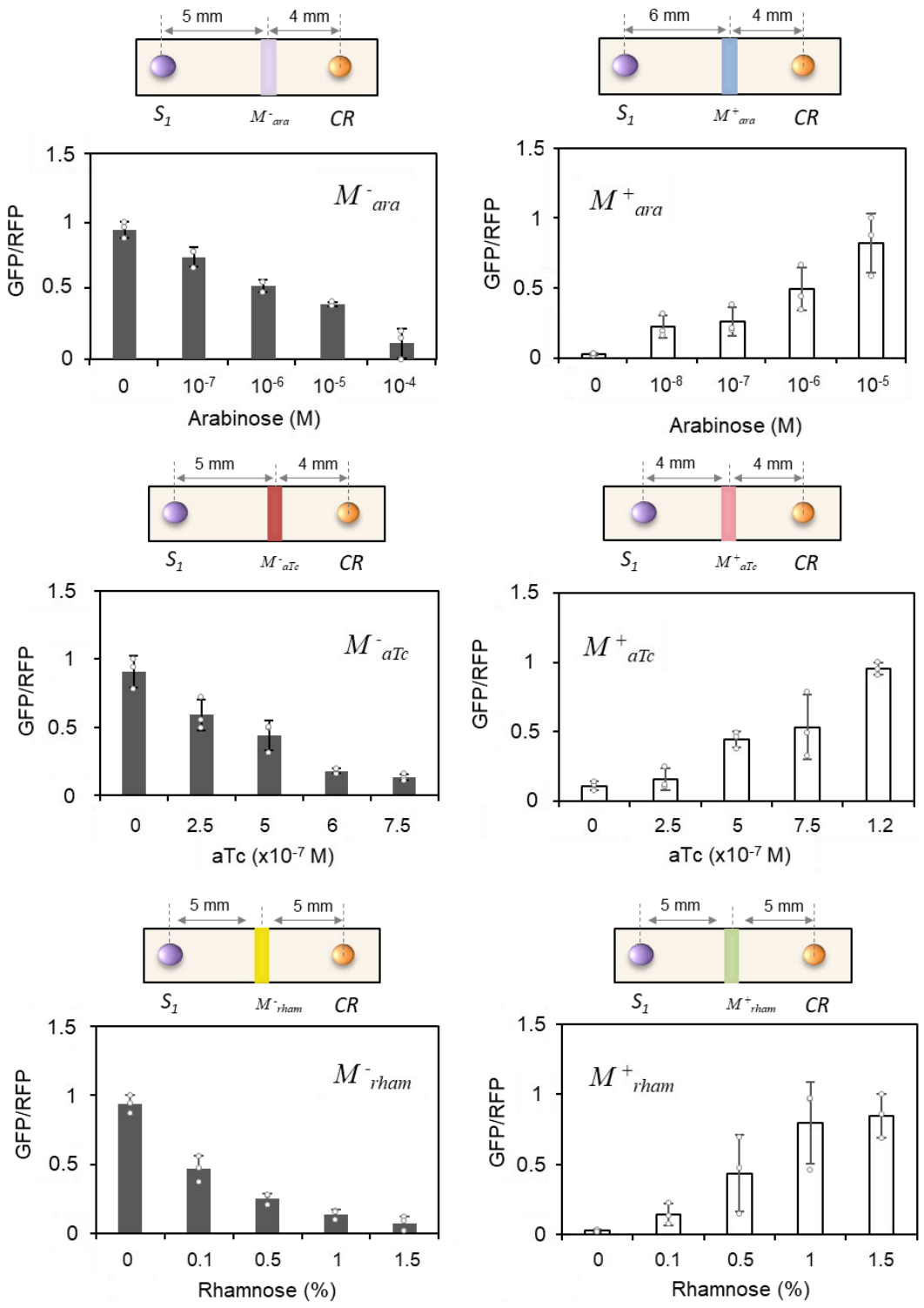
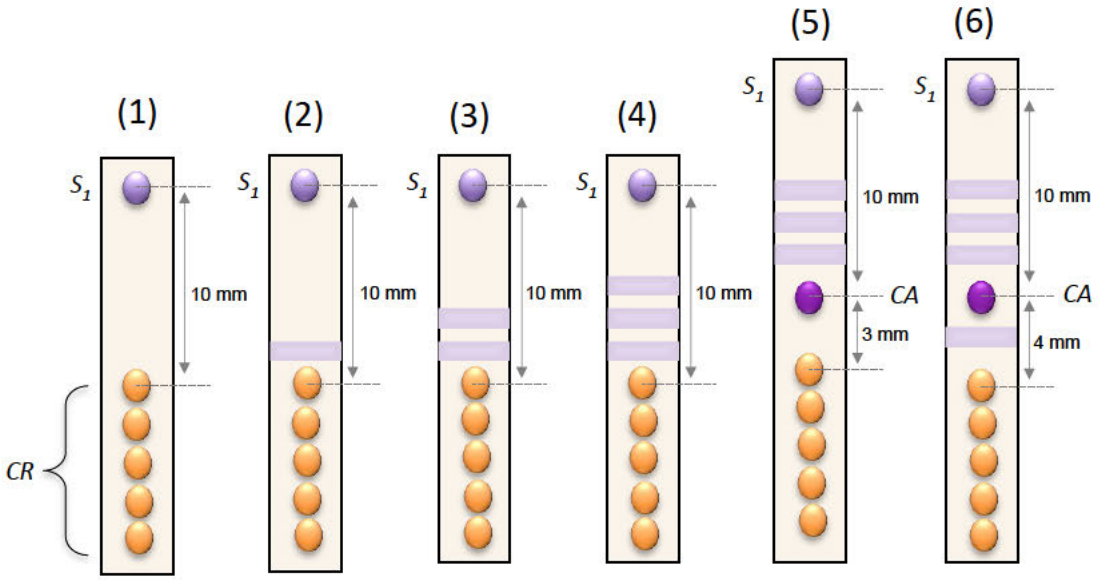


Figure 2.11. Relationship between input and output in transistor-like circuits. Output modulation in response to different input concentrations. Modulatory elements M^- (black bars) and M^+ (white bars) cells respond to the external inputs, arabinose (M^+_{ara}/M^-_{ara}), aTc (M^+_{aTc}/M^-_{aTc}) and rhamnose (M^+_{rham}/M^-_{rham}). S_1 cells produce AHL constitutively. GFP levels are normalized by RFP, which correlates with cell population. CR are the reporter cells. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

Before the creation of more complex computational devices, the effect of introducing multiple modulatory elements between S_1 and CR cells was analysed. In absence of modulatory elements, the optimal distance between S_1 and CR cells is 10mm. However, as figure 2.12 shows, when an increasing number of modulatory elements are introduced between S_1 and CR cells, a reduction of the CS levels is observed due to their basal activity of our modulatory elements, despite these modulatory elements remain inactive. However, this reduction can be restored by introducing CA cells, which allow the introduction of more modulatory elements, overcoming this limitation.

a



b

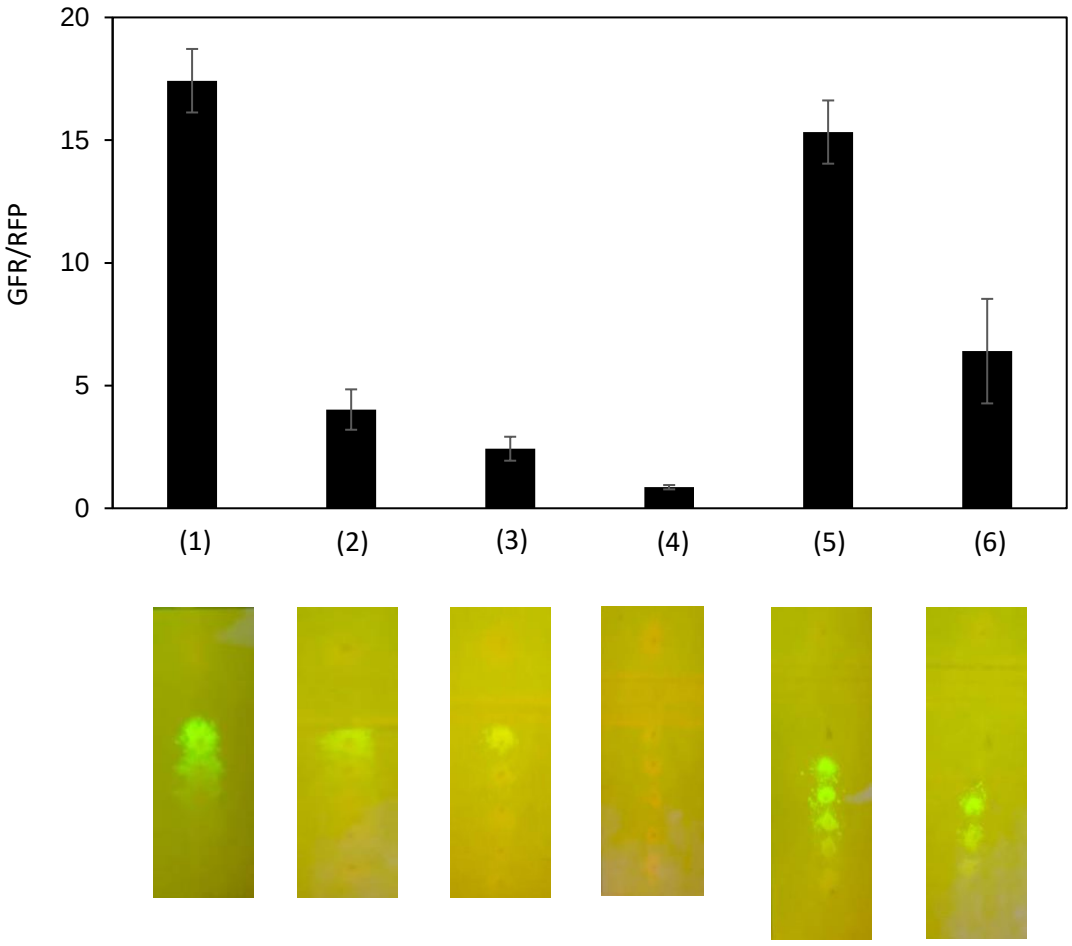


Figure 2.12. AHL signalling diffusion decay through modulatory elements. Numerical quantification of the GFP acquired by scanning the surface of paper strips, with a schematic representation of the experimental setup. To determine the signal decay due to the presence of modulatory elements, different conditions were analysed four devices involving no modulators, 1 modulator, 2 modulators and 3 modulators. Additionally, auto-amplifier cells (CA) were added to re-establish the signal decay. **a.** Schematic representation of each cellular printed pattern with the corresponding distances. S_1 is located 10mm away from CR as it is the maximum distance used in all our experiments. ***b.*** Maximum GFP expression by reporter cells depending on the number of modulators. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. Several CR dots were printed to visualize the diffusion range of AHL molecules. GFP levels are normalized by RFP, which correlates with cell population. Below the chart, different pictures of the experiments can be shown.

2.5.3 Architecture definition and scalability analysis

To develop 2D cellular circuits, we explored a general method for mapping any arbitrary truth table into a spatial pattern. Specifically, we proposed a new multi-branch architecture that is schematically represented in figure 2.13. In this topology, each input combination in a truth table associated with output 1 can be encoded in a different branch. At one end of the branch, there are CS cells, whereas at the other end, there is the output, produced by CR cells. In the middle, different bands containing modulatory cells are located. Logic inputs "1" in the truth table are encoded in M^+ cell types, which allow CS diffusion only if the input is present. Logic inputs "0" are encoded in M^- cell types, which allow CS diffusion only when the input is absent.

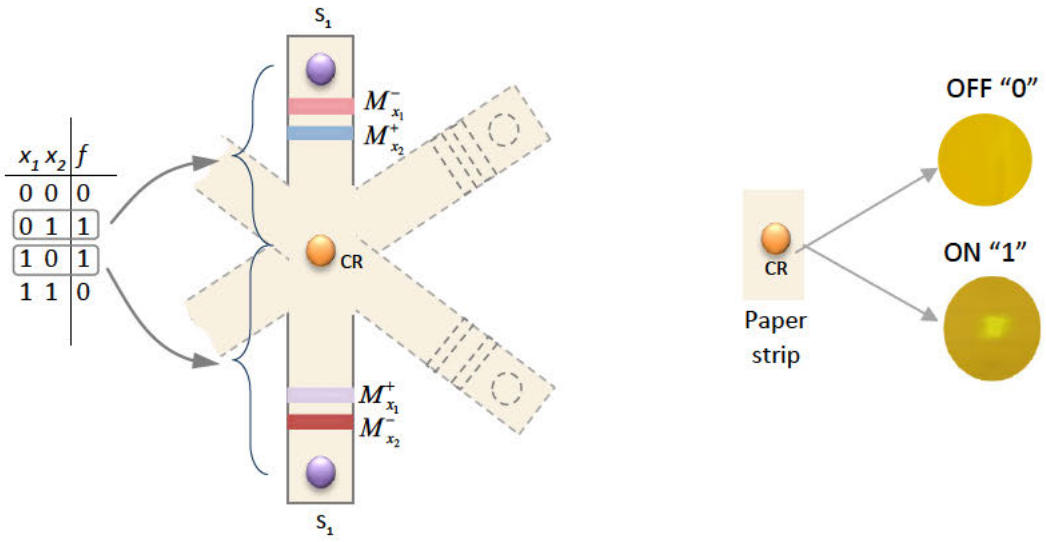


Figure 2.13. General multi-branch architecture for implementation of digital circuits. Schematic representation of the multi-branch implementation of a truth table, with its further response visualization representing the OFF "0s" and ON "1s" states.

We then analysed the scalability of this topological implementation. For circuits responding to N inputs, the maximum size of the required cell library implementing modulatory elements is $2 \cdot N$, i.e., two types of modulatory element, M^+ and M^-, for each input. Moreover, the maximum number of branches is 2^{N-1}. However, our computational analysis, shown in figure 2.14, indicates that most of the circuits require

less branches. To perform this analysis, the truth tables of all circuits responding to 2 (figure 2.14a), 3 (figure 2.14b) and 4 inputs (figure 2.14c) were simplified to its minimal configuration using the standard Karnaugh maps method[141] and directly mapped into the topological configuration, according to the method represented in figure 2.13.

These results indicate that large scalability is achievable with this multi-branched architecture.

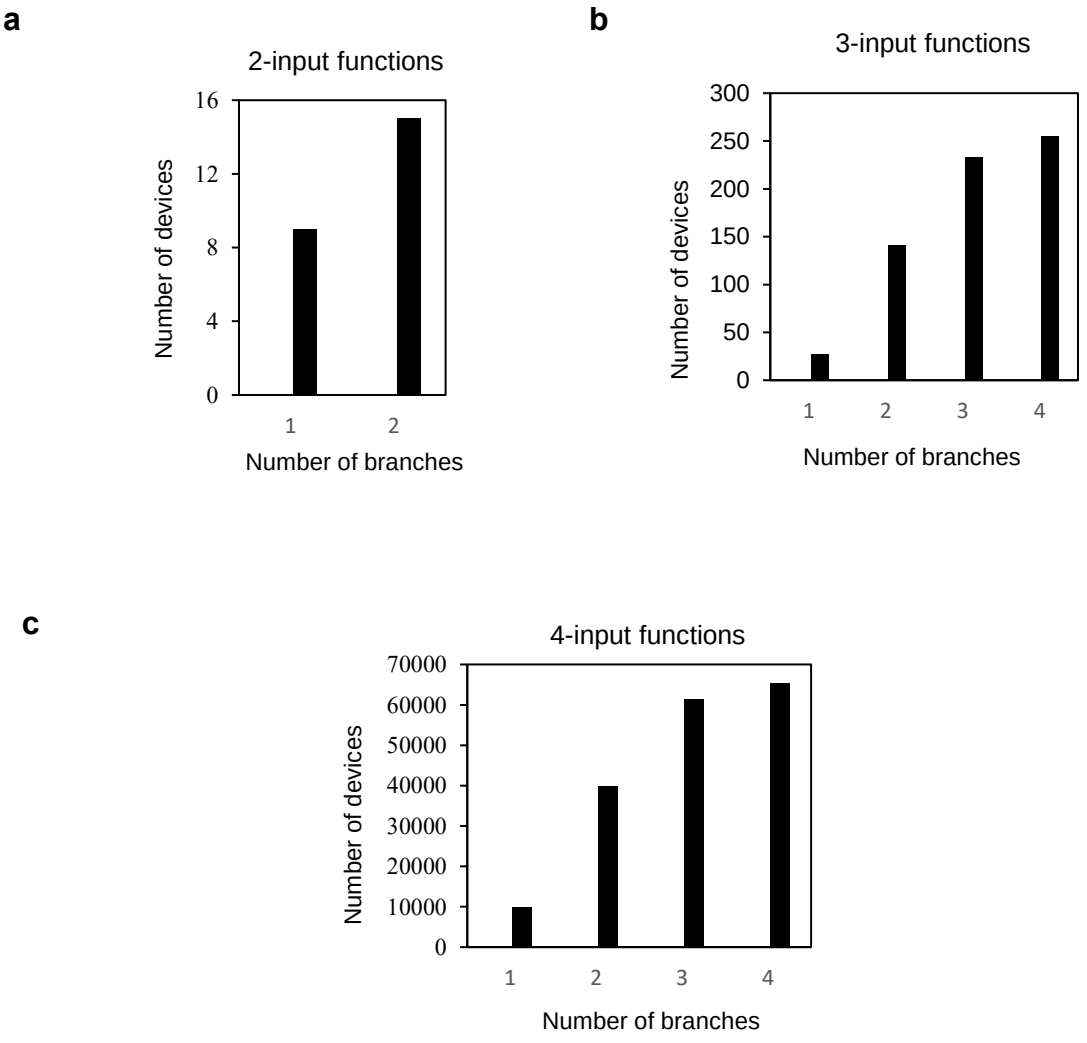


Figure 2.14. Computational scalability. Dependence of the number of different devices versus the number of different branches required. **a**. Number of branches required to implement all logic circuits responding to 2 inputs. **b**. Number of branches required to implement all logic circuits responding to 3 inputs. **c**. Number of branches required to implement all logic circuits responding to 4 inputs.

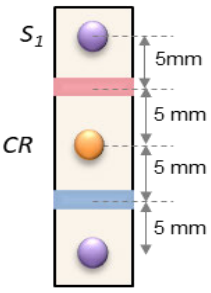
2.5.4 2-input circuits

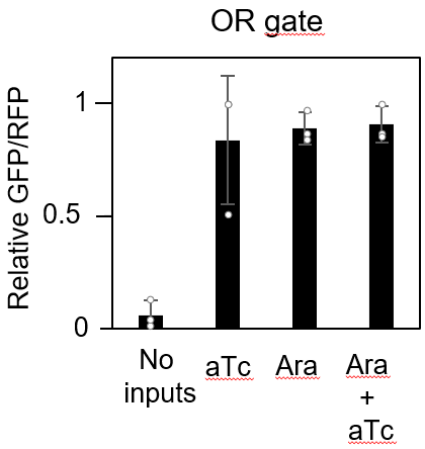
After checking the scalability of our methodology to be able to map any possible truth table, we implemented several examples of logic gates as a proof-of-principle, making use of this multi-branch architecture. These circuits, involving one or two branches, are composed of the combination of cellular elements previously described. Thus, each paper strip has a source where the CS is being secreted, some modulators to undertake the necessary computation upon each specific combination of external inputs and a collector or receiver (CR), where the final information is monitored, and thus, a “1” or a “0” is displayed accordingly.

When comparing, for example, architecture design used to implementation the OR and AND gates, by just rearranging the same cellular components, different computational behaviours can be obtained. Thus, emphasizing one of the key aspects of this work which is the low genetic complexity and number of elements needed to undertake different types of computations.

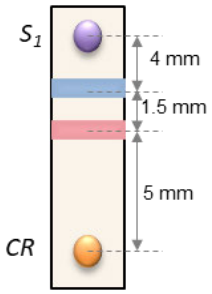
Relative GFP/RFP values obtained from these examples are shown in figure 2.15.

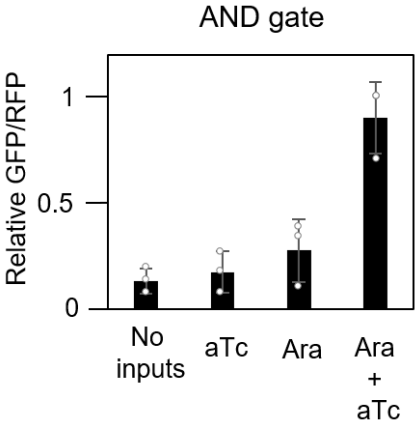
OR gate	
No Inputs	0
Ara	1
aTc	1
Ara + aTc	1



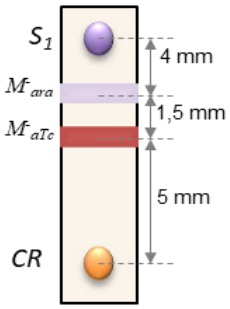


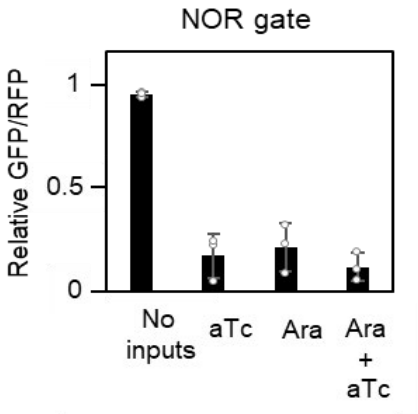
AND gate	
No Inputs	0
Ara	0
aTc	0
Ara + aTc	1





NOR gate	
No Inputs	1
Ara	0
aTc	0
Ara + aTc	0





XNOR gate	
No Inputs	1
Ara	0
aTc	0
Ara + aTc	1

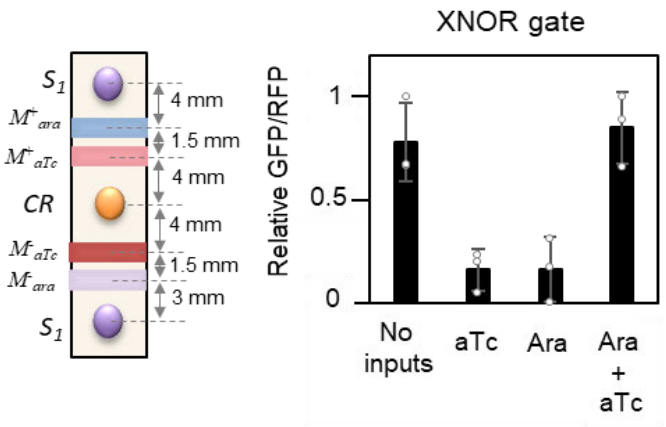


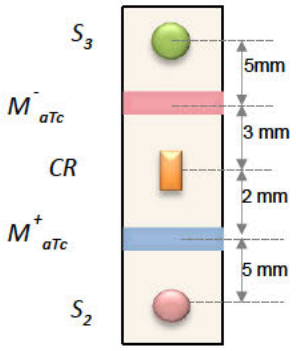
Figure 2.15. Implementation of different logic gates. Each logic gate contains its truth table on the left and a schematic representation of the cells used in each paper strip with their corresponding distance points and the response quantification on the right. Gates with two sources of S_1 (OR and XNOR gates) are circuits using two branches, while the other gates (NOR and AND gates) can be implemented with just one branch. Input concentrations are: Ara=10^{-3}M and aTc=10^{-6}M. M^{+}_{aTc} and M^{-}_{aTc} are, respectively, positive and negative modulatory cells responding to aTc. M^{+}_{ara} and M^{-}_{ara} are, respectively, positive, and negative modulatory cells responding to arabinose. S_1 cells produce AHL constitutively and CR are the reporter cells. Error bars are the standard deviation (SD) of three independent experiments. The average fold change has been obtained from the mean of ON and OFF states from each circuit. OR gate 14.31x, AND gate 6.21x, NOR gate 6.58x; XNOR gate 5.6x.

2.5.5 3-inputs circuits

Once logic gates responding to two inputs were constructed, we introduced an additional input, i.e., rhamnose, to create a 3-input device. As a proof-of-concept, we implemented a 2 to 1 multiplexer (MUX2to1). This device involves two branches producing AHL by S_3 and S_2 cells in response to rhamnose and arabinose, and different modulatory elements responding to aTc. Figure 2.16a shows the truth table describing the behaviour of this device and figure 2.16b displays the specific spatial topology. Figure 2.16c shows the circuit response upon different input combinations. As seen in this figure, the device behaves according to the truth table, implementing the expected response.

a

aTc	Rham	Ara	Output
0	0	0	0
0	0	1	0
0	1	0	1
0	1	1	1
1	0	0	0
1	0	1	1
1	1	0	0
1	1	1	1



b

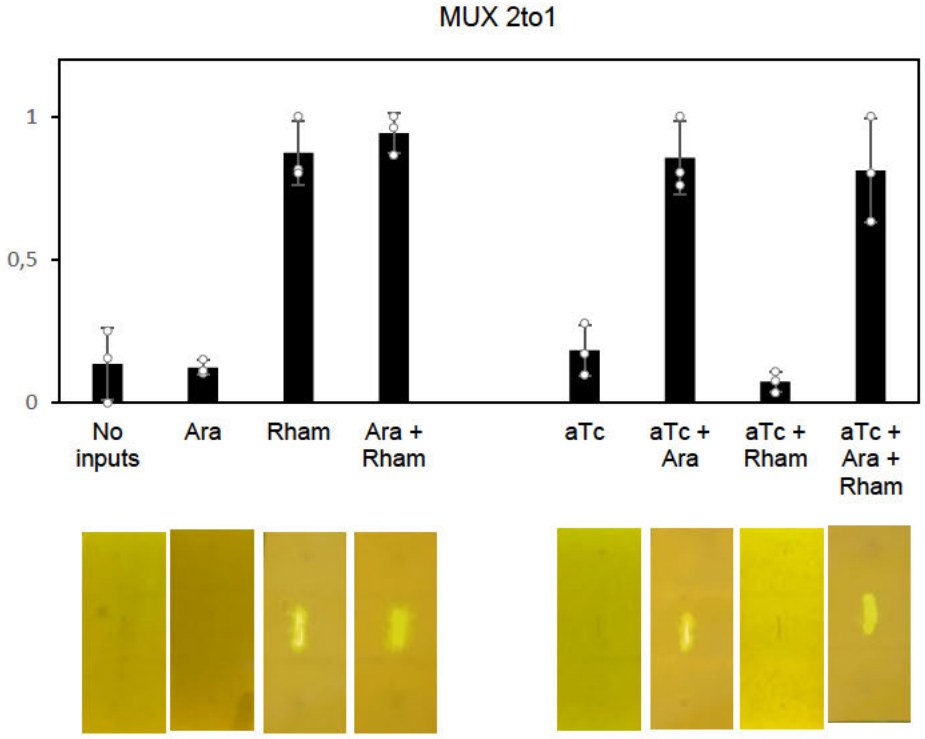
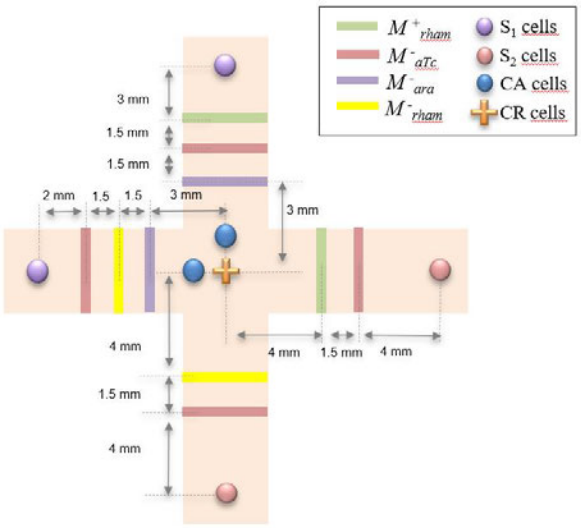


Figure 2.16. Multiplexor 2to1. a. Truth table of the 2to1 multiplexerand spatial pattern of cells encoding the multiplexer device with their corresponding distances. M^+_{aTc} and M^-_{aTc} are, respectively, positive, and negative modulatory cells responding to aTc. S_2 cells produce AHL upon arabinose induction and S_3 cells produce AHL upon rhamnose induction. CR are the reporter cells. **b.** Multiplexer response upon input of the indicated combinations. Input concentrations are: Ara=10^{-3} M, aTc=10^{-6} M and Rham=1.5%. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. The average fold change has been obtained from the mean of ON and OFF states from each input combination. Multiplexor 2to1 fold change: 6.76x.

To explore more complex topologies, an even parity bit circuit was also implemented. This circuit, responding to three different inputs, involves four different branches with up to three modulatory elements per branch. Due to this large number of modulators, an auto-amplifier cell (CA) was introduced close to the output cells CR to restore AHL levels. Figure 2.17a shows the truth table describing circuit's logics, which is translated into a printed pattern (figure 2.17b). Experimental results are shown in figure 2.17c, indicating a proper circuit response.

a

Ara	aTc	Rham	Output
0	0	0	0
0	0	1	1
0	1	0	1
0	1	1	0
1	0	0	1
1	0	1	0
1	1	0	0
1	1	1	1



b

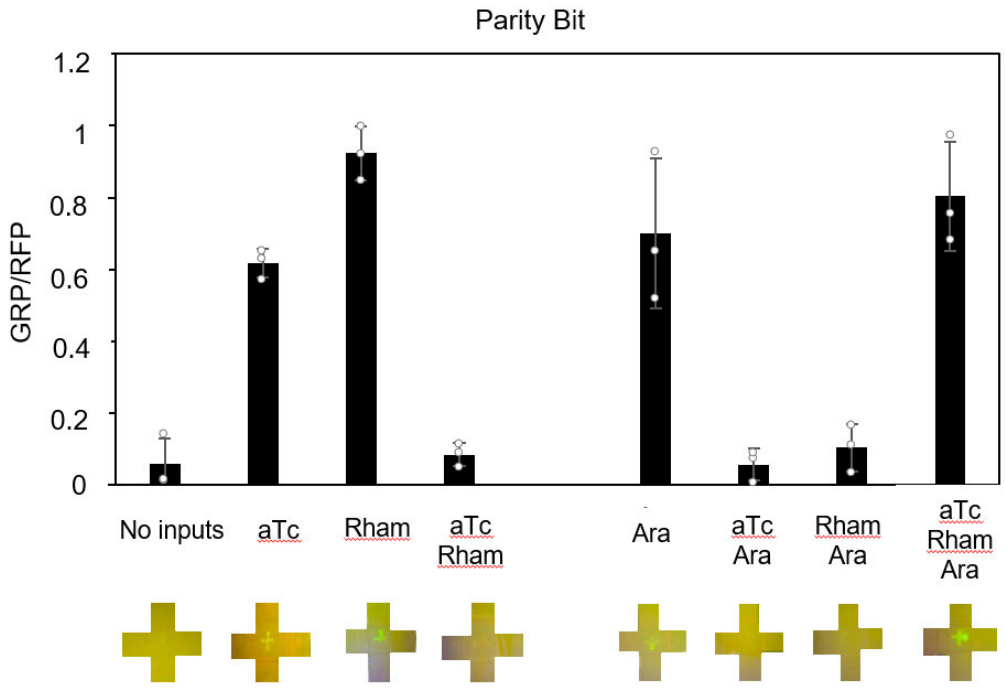


Figure 2.17. Parity bit. a. Even parity bit truth table and spatial pattern of cells encoding it. This circuit involves four branches, with the final output in the middle. The output cells CR were printed as a cross to visualize from which branch comes dominantly the AHL signal. M^{+}_{aTc} and M^{-}_{aTc} are, respectively, positive and negative modulatory cells responding to aTc. M^{+}_{rham} and M^{-}_{rham} are, respectively, positive, and negative modulatory cells responding to rhamnose. S_1 cells produce AHL constitutively and S_2 cells produce AHL upon arabinose induction. **b.** Response upon input of the indicated combinations. In this circuit, due to the number of modulatory elements per branch, auto-amplifier cells (CA) were incorporated close to reporter cells (CR). Input concentrations are: ara=10^{-3} M, aTc=10^{-6} M and rham=1.5%. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. The average fold change has been obtained from the mean of ON and OFF states from each input combination. Parity bit fold change: 10.47x.

2.6. Expanding the library of engineered cells to perform analogue computations

Not only digital, but also analogic computations were considered to prove the versatility of printed circuits. For it, an example of an analogic circuit, i.e., a band-pass filter, was implemented using the same elements described in Figure 2.18, except that the cell type S_1 (constitutive production of AHL) was replaced by S_2 (arabinose-inducible production of AHL). This allows the introduction of additional modulatory element as AHL production depends on the external input, in this case arabinose.

At low arabinose concentrations, AHL levels are low due to the low production in S_2 cells. The combination of low AHL synthesis with the reduction of AHL due to its diffusion does not allow AHL-induced output production by CR cells. In contrast, at high arabinose concentrations, although the higher AHL secretion, it also induces the production of Aiia by the modulatory element (M^-_{ara} cells) located between S_2 and CR cells, which degrades the diffusing signal and therefore, blocks the AHL flow and the output is not produced. However, at intermediate arabinose concentrations, the level of AHL secreted from S_2 cells is sufficient for CR activation but the arabinose concentration is not high enough for M^-_{ara} activation.

Thus, AHL concentration induces output production in CR cells. Figure 2.18 shows the GFP/RFP levels at different arabinose concentrations, indicating a clear band-pass response.

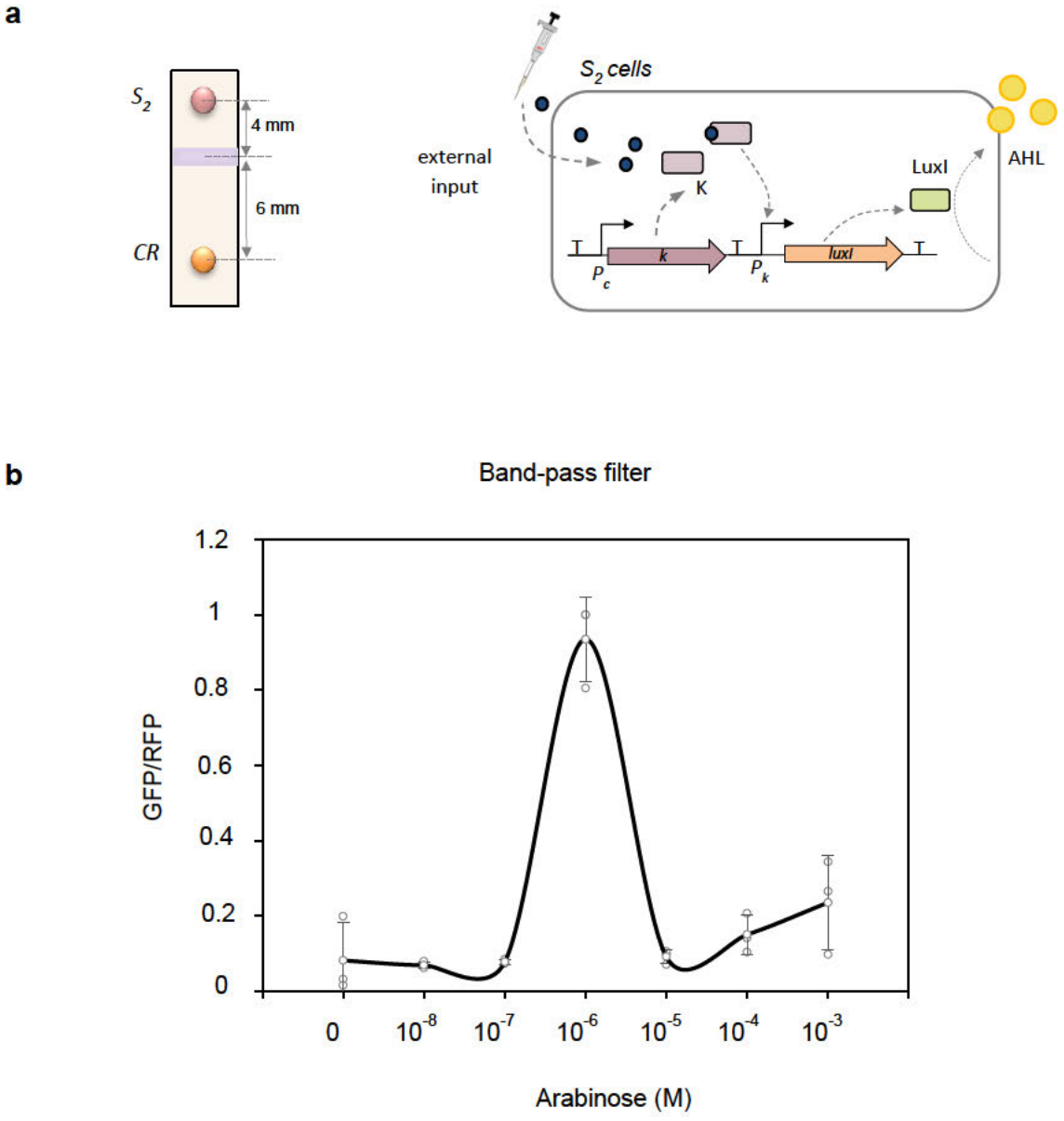


Figure 2.18. Band-pass filter circuit. a. Band-pass filter implementation is outlined by a schematic representation of the cells used for the paper strips and their corresponding distance points. M^{-}_{ara} are negative modulatory cells responding to arabinose. S_2 cells produce AHL upon arabinose induction and CR are the reporter cells. **b**. Circuit response upon increasing concentrations of the external input, i.e., arabinose. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

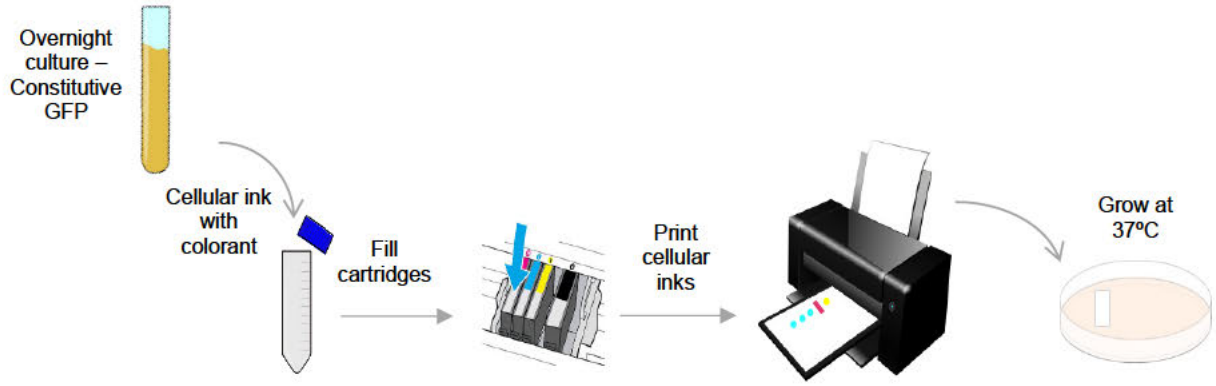
2.7. Exploration of circuit properties

2.7.1 Autonomous assembly and circuit flexibility

The next issue addressed was the automation of the proposed methodology for circuit printing. In this study, we explored the construction of different cellular devices on paper surfaces following a pre-defined theoretical pattern. To make this process autonomous in an industrial scale, we explored different methods. The first approach consisted of trying to print patterns using an InkJet printer, giving each cellular module, i.e., source, modulator, or reporter cells a pure colour from the CMYK model (Cyan, Magenta, Yellow or Key/Black) using the defined topology we used among this work. Afterwards, we used refillable ink containers and we filled them with our engineered bacteria, a thickener, in this case, glycerol and food dye.

Interestingly, cells were able to grow and express their genes correctly. However, we had some problems regarding cellular ink contamination, as we could observe some colony contamination in our printed pattern. Figure 2.19 shows some of the trials we performed.

a



b

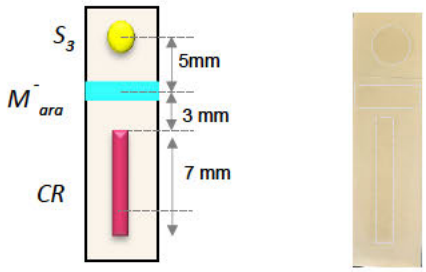
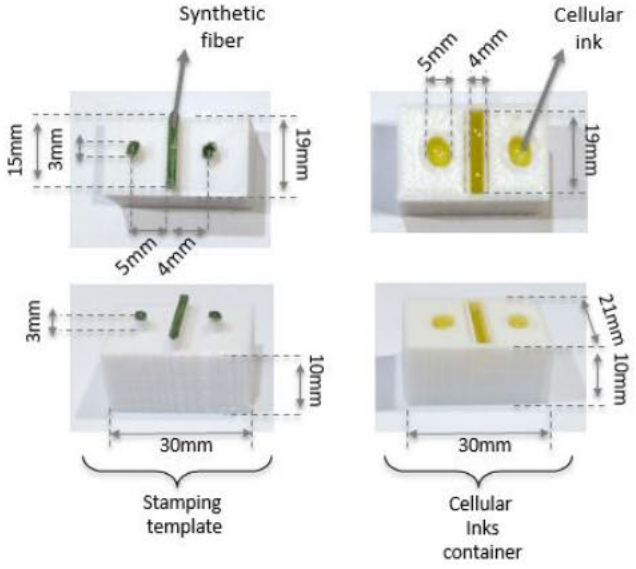


Figure 2.19. Printing process. As shown in the diagram, inks were fabricated from an overnight culture with colorants, thickeners, and antibiotic to avoid contaminations. Then cartridges were filled, and a template was printed and grown in agar plates at 37 ºC.

After exploring this method and realising some of its challenges, we realised that stamping could be a good alternative to avoid these contaminations and make the process more robust and easier to implement. For it, we used a container filled with different cellular inks which were then stamped into paper. Stamping offers numerous advantages over previous methods for cell printing, such as 3D printers[142]. For instance, stamping is faster than 3D printing, does not require complex printing devices, and templates can be easily customized, which makes stamping suitable for industrial production potentially at very low cost and with minimal requirements.

The figure below (Fig.2.20) shows the template it was used to stamp a simple circuit, i.e., the first transistor-like circuit with one modulator (M^-_{ara}). Figure 2.20b shows the obtained circuit response.

a



b

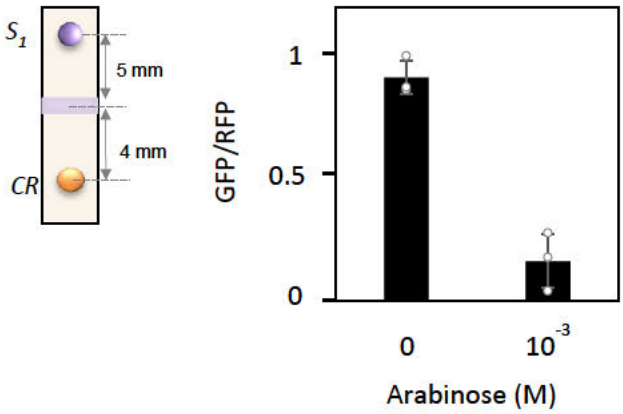


Figure 2.20. Printed circuit implementing a transistor-like device. a. Mapping of a general transistor architecture on a cellular printed pattern obtained using a stamping template. Similar to the transistor architecture, the cellular pattern is composed of three main components: source (S_1 cells), gate (M^- cells) that responds to external inputs and a drain (CR cells) as the final output responding to the presence of the carrying signal (CS). **b.** Response quantification. In the absence of external input, i.e., arabinose, the CS encoded diffuses along the surface, inducing GFP expression in reporter cells CR. In the presence of 10^{-3} M arabinose (Ara), the modulatory element M^-_{ara} produces the AHL cleaving enzyme Aiia, which degrades the CS. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. Experiments are performed on paper strips. The average fold change is 5.2x.

During the stamping procedure, two templates were used, one filled with cellular inks (i.e., different engineered cell types) and another one with the circuit template recovered with a synthetic fibre to trap the inks. This stamping template was soaked with the different cellular inks and stamped on a paper surface according to the topology being designed, codifying the computation to be performed. Finally, the stamped paper was deposited on an LB-agar plate mixed with the different inputs and was subsequently incubated at 37 ºC for 24 hours.

Figure 2.21 shows a schematic representation of this pipeline.

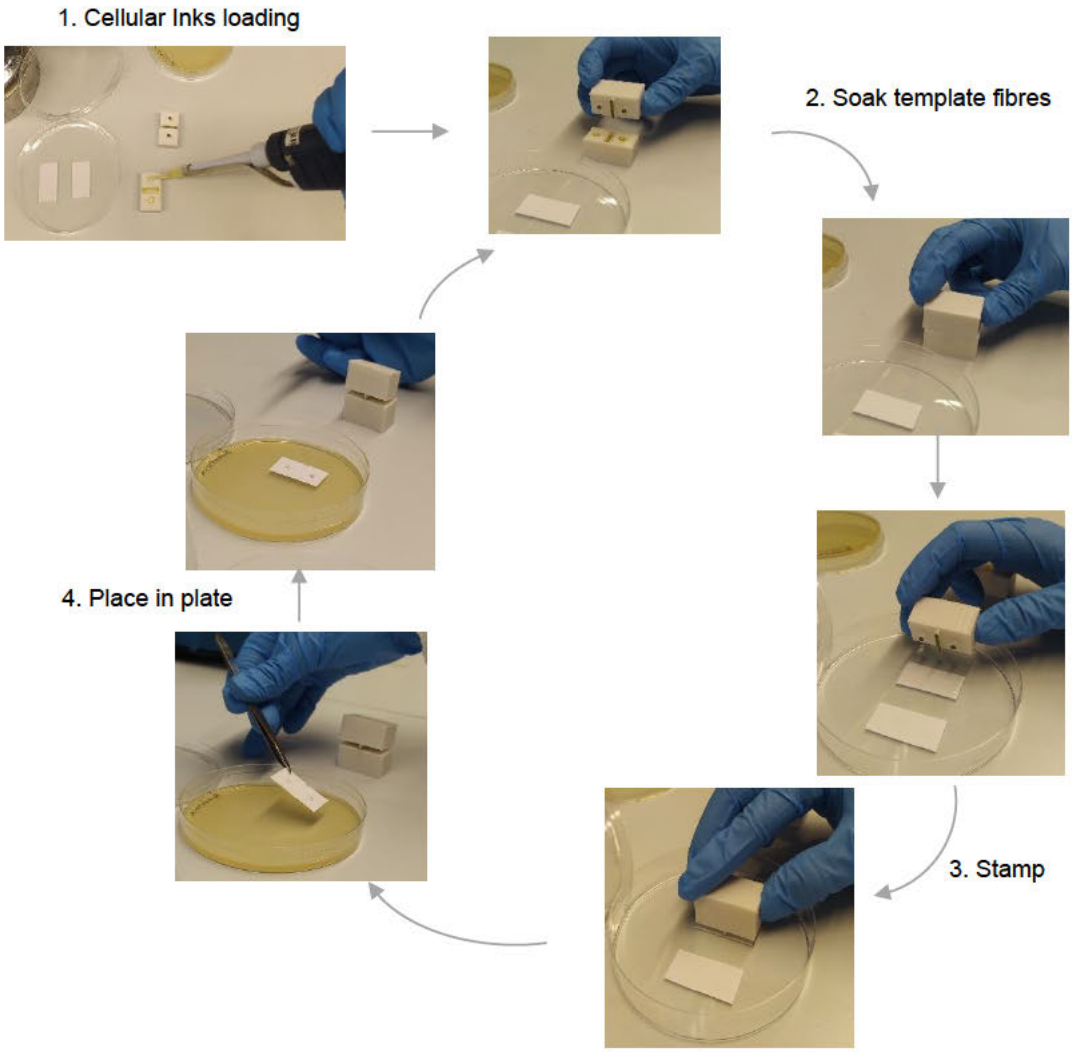


Figure 2.21. Stamping process. Pictures visually describe the process made to stamp our circuits. Prototypes are filled with different cellular inks; the template is then soaked, and the circuit is stamped in a paper strip. This strip is then placed on an LB-agar plate to incubate at 37ºC.

2.7.2 Scalability

As mentioned previously, the use of stamping templates enables the use of a general mould, adapting it to each specific circuit. It should be pointed that previous experiments were carried by manual distance adjusting to determine the proper connection between elements. Once these distances are established, stamping patterns can be assembled for circuit automation.

An analogy between our theoretical model and its stamping template is displayed in figure 2.22.

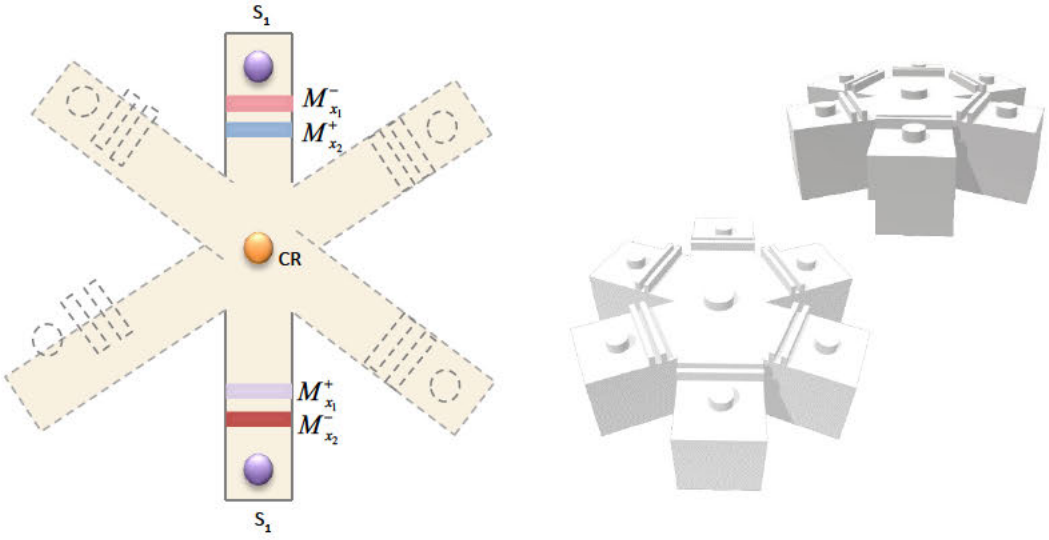


Figure 2.22. General stamping prototype template. This template represents the general architecture used to map any circuit, using this multi-branch topology. Additionally, 3D designs for this multi-branch approach are also displayed to allow scalability and reproducibility.

It should be pointed that all circuits have been optimised manually to determine the optimal distances between all circuit elements. Although once these distances are established, multiple copies of the same system can be produced, this first process of optimisation should be automatized to create an improved methodology.

2.7.3 Other fabrics

Despite this study focuses on the use of paper as a substrate, other additional materials can be used for printed circuits. As a proof-of-principle, we have printed the same circuit shown in figure 10a on nylon fabric. As seen in figure 2.23, the circuit printed on nylon fabric has also the expected performance upon the presence or absence of external inputs, i.e., arabinose.

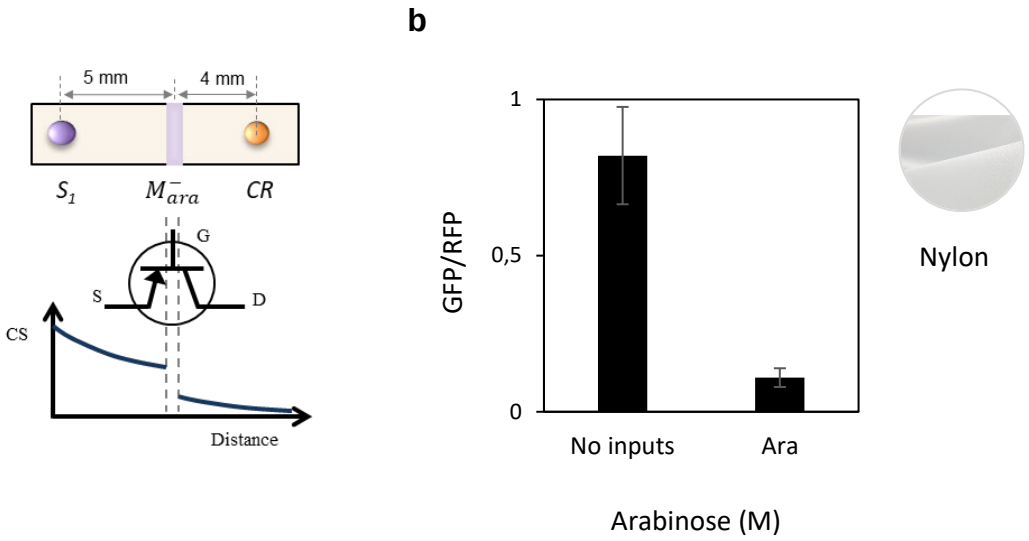


Figure 2.23. Transistor-like architecture printed on nylon fabric. a. Mapping of a general transistor architecture on a cellular printed pattern composed of three main components: a source (S), a gate (G) that responds to external inputs and a drain (D) as the final output responding to the presence or absence of our carrying signal (CS). **b.** Circuit response. In the absence of external input, i.e., arabinose, the CS encoded in the production of AHL molecules by S_1 cells diffuses along the surface, inducing GFP expression in reporter cells CR. In the presence of 10^{-3} M arabinose (Ara), the modulatory element M^-_{ara} produces the AHL cleaving enzyme Aiia, which degrades the CS. Experiments are performed on a nylon fabric. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD. The average fold change is 7.5x.

2.7.4 Stability

The next crucial aspect to be analysed was circuit stability. Most biological circuits work during a specific, usually short, time window, so circuits must be discarded. Moreover, the preparation of them is usually complex.

Here, we explored different options to make our devices capable to work after a certain storage time and conditions.

We proved that paper circuits could be either used directly to perform the desired computation or could be stored, in the fridge at 4ºC or frozen at -80ºC, for future use.

For circuits stored at -80 ºC, the same principles were used but, in this case, a 20% v/v glycerol was used for the mixture, rather than the agar. Glycerol cryoprotects cells deposited on paper, so circuits can be thawed and used later.

To evaluate the temporal stability of the transistor-like circuit described in figure 2.9, we analysed the performance evolution of this printed circuit along time, storing them at 4ºC for several days.

Experimental results, shown in figure 2.24, indicate that circuits stored at 4º C exhibit a progressive reduction of the fold-change over time, i.e., up to 10 days. Despite this reduction, the device exhibits a proper behaviour. On the other hand, frozen circuits do not exhibit significant changes in their performance, maintaining an optimal functionality, leading to larger durability.

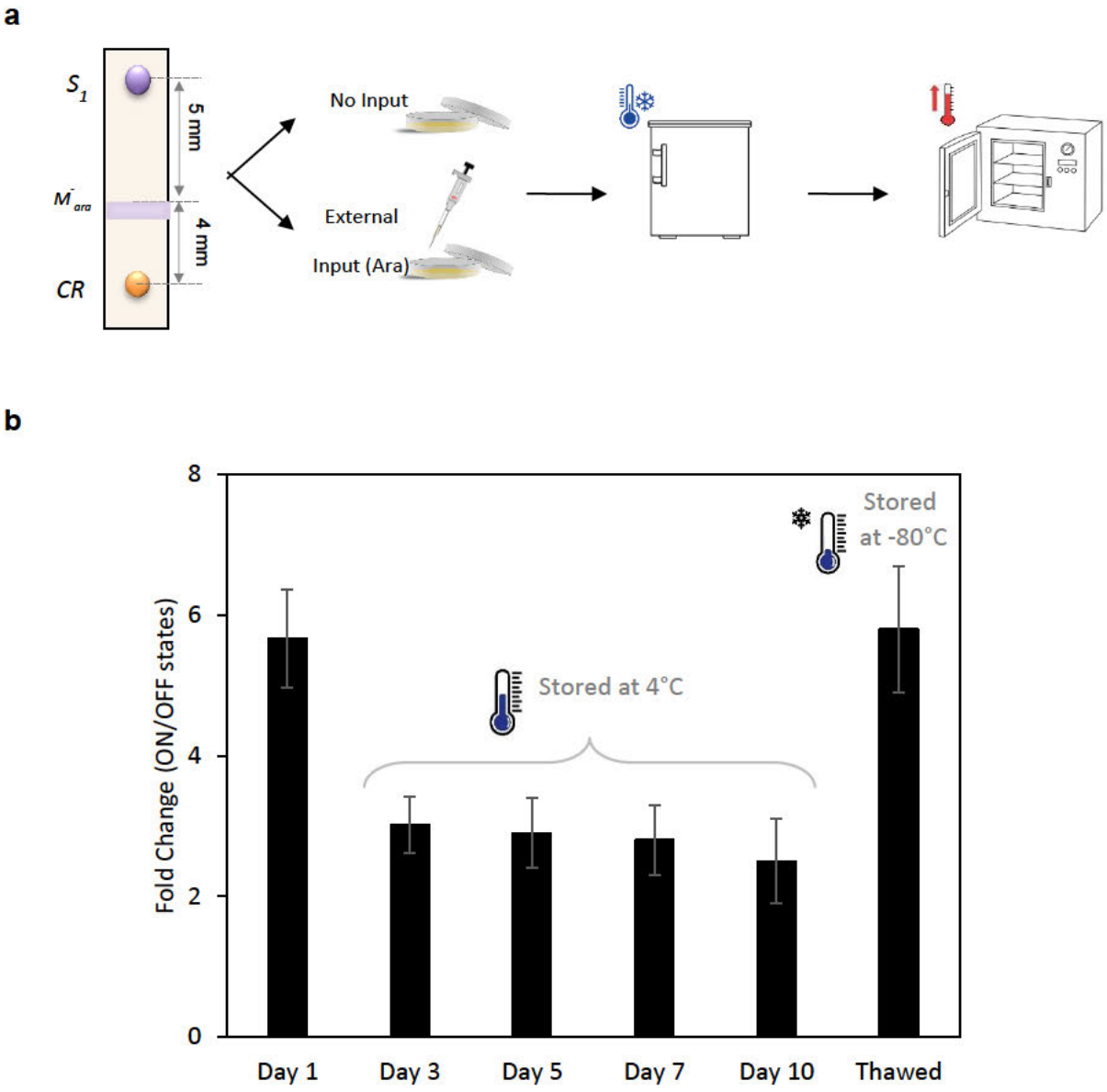


Figure 2.24. Circuit stability and storage. Temporal stability of printed circuits. As a case study, a simple transistor-like device (identical to figure 2.9) was analysed. a. Description of the experimental procedure. Circuits were stored on the fridge at 4°C. Every 2 days, circuits were grown at 37°C and measured after 24h. b. Experimental quantification of circuit response. Despite the fold change reduces when circuits are stored in the fridge, differences between the ON/OFF states are still significant after 10 days. Circuits frozen at -80ºC do not show effects on their performance and maintain their functionality, once thawed. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

2.8. Future work

However, this project aims to go beyond the creation of multicellular printed circuits for specific purposes and explores the creation of circuits for general purposes that can be reprogrammed to perform a specific computation. Here, taking inspiration from current electronic FPGAs (Field Programmable Gate Array) technologies, we plan, in the future, to create reprogrammable devices. FPGAs are an array of programmable elements and a hierarchy of reconfigurable interconnections that allow the elements to be wired together in different configurations. Hence, FPGAs can be reprogrammed allowing flexible reconfigurable computing as performed in computer software.

Taking inspiration from this idea, we plan to create devices consisting of a printed array containing the whole set of cell types configuring the functional complete set. In consequence, all possible computations to be performed could be implemented in this kind of devices. However, our approach has a significant difference with respect to electronic FPGAs architecture. Instead of reprogramming the matrix of interconnections, the printed cells will be inactive, i.e., will not respond neither to the carrier signal nor the external inputs. Differently, these cells will contain an optogenetic circuitry that on the presence of light, it will induce irreversible changes in the DNA, i.e., using recombinases, activating the cells that have been exposed to the proper wavelength. By applying a proper pattern of light spots, the general inactive device will change to an active specific configuration able to perform the desired computation.

In summary, the main goal of this project is the first proof-of-concept of a novel and ambitious science-to-technology breakthrough for creation of complex living computers reaching a *Technology Readiness Level* 3. This disruptive approach will overcome current limitations and can significantly contribute to the advance of living technologies.

2.9 Discussion

The language of information processing is widespread in biology. From DNA replication to nerve impulses in the brain, biological systems are frequently known for storing and processing data, and even as performing intrinsic computation. Biological and computational processes share many similarities, such as: the encoding of process data in proteins; signal transduction from input to processed output [143]; cells mimicking computers [144]; correlation between computer networks and biological distributed systems [145]. Computational biology aims to model biological systems computationally, to better understand their basic processes, and also for producing biological systems artificially[146].

Modern synthetic biology has been defined as an engineering discipline to design new organisms as well as to better understand fundamental biological mechanisms. However, success is still largely limited to the laboratory and transformative applications of synthetic biology are still in their infancy[147].

Biological systems perform computations at multiple scales. Engineering metaphors have been used to provide a rationale for modelling cellular and molecular computing networks and as the basis for their synthetic design, which attempts to engineer biological systems to perform desired functions[148]. However, major constraints when mapping those theoretical models in the living world occur, such as the wiring problem. Although wires are basic cables that interconnect elements in electronic devices, they must be different when using synthetic biology designs, as each connection depends on an orthogonally different wiring molecule. Moreover, in most cases the designed molecular systems cannot be reused for other functions as it is specifically designed to satisfy a specific context with specific input responses[149].

The main goal of this work was to design a new methodology to create simple, scalable, robust, and cost-effective genetic circuits to enable any type of computation. Standard designs use different layers for information processing, i.e., input layer, processing layer and output layer. In our approach, we are using a completely new framework based on computationally active surfaces that can be configured based on different topology designs, where no distinction between input and processing layers

are considered. Then, by placing our main engineered units, i.e., genetically engineered constructs, in different spots on the surface, different desired behaviour can be obtained.

Interestingly, computational properties of intracellular biochemical networks have already been extensively characterized. What has so far not received systematic treatment is the importance of space in intracellular computations. Spatial network computations are required for essential spatial processes (e.g., polarization, chemotaxis, division, and development) but also to simply computation as an extra dimension is used to expand the computational power of the network[37].

Connected to space, multiple studies have demonstrated that multicellular embodiments are optimal to implement complex computational devices. However, co-culturing different cell types in the same medium induces the emergence of problems related to competition for nutrients, which can compromise large temporal stability. This problem has been overcome introducing this concept of multicellularity with spatial segregation and distributed computation[109].

When analyzing existing work done in this field, we can observe that most methodologies require multiple wiring molecules coexisting limiting scalability and system robustness. Other approaches only require a single wiring molecule but the experimental setup is complex and the composition and stability of the multicellular consortia must be carefully controlled[30], thus, not being optimal for possible future applicability and implementation outside a laboratory environment.

On the other hand, different approaches based on cell-free systems[150] are suitable for paper-based devices and are thought to be suitable for its technological transference to cover market needs. Nevertheless, the limited number of biochemical reactions available in these systems is a limiting factor for the creation of complex devices involving multiple circuit components. Moreover, these systems require complex storing conditions for its stability and are expensive to produce.

Complementary to this inefficient methodologies, when considering market applications, engineered organisms find themselves in environmental contexts where

fluctuations of physical variables occur, such as changes in temperature and osmolarity, population density and unexpected interactions[27] which are not occurring in controlled environments such as laboratory facilities.

For all these reasons, the present results are just a small piece of the potentially far-reaching implications and possibilities of this approximation. The methodology presented in this study combines the benefits of the previous approaches but overcoming some of their limitations, namely only a single molecule is required for computation and different cell types can growth separately, avoiding competition effects. Additionally, we have also demonstrated that with a fixed number of computational units, i.e., CS, modulators and receivers, many types of computations with different complexities and different behaviours can be obtained, even obtaining both digitalized and analogic responses. Moreover, we have also explored the potential methodology scalability, being a relevant aspect of this work, not representing a limiting factor as in many previous studies.

Although our system works for three inputs, future work should be devoted to i) scaling up this system to enlarge the possible computations, ii) automatize the stamping of the complex circuits for large scale industrial production and iii) reduce time response.

In summary, printable cellular circuits could represent an interesting alternative for paper-based devices with appealing potential advantages such the possibility of producing low-cost cellular devices based on a simple experimental setup methodology. Further development is required to explore biological richness to solve real-world problems and meet further market needs such as personalized medicine, sustainable energy-rich molecules for fuels, bioremediation, or food supplies among others.

Achievements:

- *Premio Universidades Excelentes –* ***Best Research Award*** *(El Mundo)*

- ***Publication*** *in Nature Communications*

CHAPTER 3

The Art of Living Biosensors – Section 1

Biosensor-related publications were mostly inexistent in the 20th century. The early era of biosensing was first emerged with the invention of the oxygen electrode in 1955 by Clark[151]. From this first invention, subsequent modifications led up to the next publication in 1962, developing the first glucose sensor and the enhancement of electrochemical sensors, e.g., polarographic, potentiometric and conductometric, with enzyme-based transducers. Clark transferred the technology to a company to start commercializing the first glucose biosensor in 1975. Therefore, although the many advances occurred in this field, it is still quite new, and many changes are yet to occur.[152]

Nowadays, there are multitude of instruments which employ the term biosensor in different areas of the world as diagnostic tools in point-of-care testing. However, the realization of cost-effective, accurate, specific, and user-friendly devices is limited to a small number of well-established sensors. In most cases, miniaturizing is a main concern, together with the production cost and the time-consuming performance. Thus, many of them are used in laboratory infrastructures, using expensive and sophisticated equipment, not being able to be deployed in relevant environments[153,154].

Cell-based biosensors have been developed as potential alternative to already existing analytical devices, as these can detect a wider range of molecules in various areas. The most relevant areas have been bioproduction and medical and environmental monitoring[155].

Environmental monitoring has been a relevant focus because biosensors can give information not only on the presence of pollutants but also on their bioavailability, which is important when considering the impact of the pollutant on the environment. Cell-based biosensors also offer the possibility of remote testing, which is a significant advantage when testing for dangerous materials such as explosive residues from mines[156].

For medical applications cell-based biosensors offer faster diagnostics than traditional methods, where culture of the infectious agent is commonly required as well as

transportation to a testing lab. More recently, with the rise of interest in point-of-care testing and health monitoring, wearable cell-based biosensors have been developed to the proof-of-concept stage[157,158]. The development of technologies such as microfluidics has also meant that biosensors can be used in a high throughput manner which is highly important for identification of new drugs[159] or drug resistance[160,161].

Cell-based biosensors also allow the detection of a pathogen to be linked to downstream processes such as the production of a treatment[162].

Food safety applications have also been studied but less, although there is an increasing demand for improvements in global food safety. The main focus has been on the detection of molecules of interest which must not to be present in food, commonly allergens and pathogens[163]. However, it is a challenge to detect and identify these harmful organisms in a rapid, responsive, suitable, and effective way[164] for its prevention and recognition of problems related to health and safety[165,166].

Some interesting bio-detection systems are displayed below, depicturing the potential of making use of already existing elements from nature.

- Cascaded amplifying circuits enable ultrasensitive cellular sensors for toxic metals – tuning mercury and arsenic biosensors to achieve better responses by modulating promoter strengths, amplifiers, and receptor binding proteins.
- Probiotic for treating cholera, avoiding vibrio cholera colonization in the intestine thanks to the present of bacillus lactis [162]
- Ingestible biosensor for gut bleeding [167]
- N-acyl-homoserine-lactones detection systems. The role of bacteria in the pathogenesis of several diseases, including gastrointestinal (GI) disorders, is well established. Moreover, rather recently bacterial quorum sensing has been implicated in the onset of bacterial pathogenicity. Thus, it has been hypothesized that the signalling molecules involved in bacterial communication may serve as potential biomarkers for the diagnosis and management of several bacteria-related diseases.[168]

OBJECTIVE 1

The main goal of this chapter is to re-design the way biosensors have been thought, to open the possibility to create living devices capable to work out of the lab in relevant environments.

For it, this chapter aims to create improved and universal biological sensor architectures for a broad range of detectable molecules (i.e., from chemicals to microorganisms) which can be used on-site as point of care (POC) devices and are suitable for analyte identification and quantification.

*In the **first** section, a new concept will be explored, using space and diffusion gradients to quantify chemical molecules for easily deployable sensors for signal detection and quantification.*

Using previous knowledge on 2D patterning and multiple input combination presented in Chapter 2, we seek to be capable to create easily marketable biosensor designs, which can visually and electronically quantify the concentration of analytes of interest, as well as give visual insights on the presence or absence of other type of molecules which are potentially fatal for humans

As a proof-of-concept, we aim to create two types of biosensors, one to detect and visually quantify chemicals, i.e., mercury, covered in this first section, and another one to continuously monitor microbiologic agents. In this case, two scenarios are explored for the detection of human-specific pathogenic microorganisms, i.e., *V. cholerae* and its further implementation in underdeveloped countries, and animal-specific pathogens, i.e., main *Vibriosis* affecting fish farms as a case study.

This last example is the starting point for the creation of a spin-off company from the University Pompeu Fabra.

In this first section, the main objectives are the following:

1. Creation and characterization of engineered cells capable to detect chemicals of interest, i.e., mercury ions as a proof of concept

2. Methodological approach to visually quantify chemical abundances

3. Prototype assembly and testing

3.1 Biosensor market opportunity

Chronic diseases and conditions such as heart diseases, cancer, type 2 diabetes, stroke, obesity, and arthritis, are some of the most common, prevalent and at the same time, costly global health problems, which prompt the development of biosensors to have an optimal early detection and the acceptance of end-users/patients. However, although these needs, the technology has a slow rate of commercialization due to the high costs and technological transference difficultires[169].

Nevertheless, recently, demand for simple, fast, disposable, user-friendly and cost-efficient devices for multiple detection purposes has widely increased, implying a significant raise in these product sales. The global biosensors market size is expected to reach $34.3 billion by 2025, raising at a market growth of 8.6% CAGR (Compound Annual Growth Rate)[170]. The main factors influencing this growth rate are the high demand for diagnostic devices, the fast-technological advances, and the various applications in the medical field, but also in many other fields. The most common biosensors are for glucose monitoring or for pregnancy tests. However, several other applications such as drug detection, food safety and DNA testing are arising[171].

It is well-known that early and precise diagnosis is a key aspect for the successful prognosis of a disease, implying a better survival rate within patients. For this reason, this rapid diagnosis tests are gaining investment form private companies, venture funding and governmental entities are giving support to the development of new products for this detection to improve and extend to different sectors.

The rest of non-medical applications, i.e., environmental monitoring, food industry, fermentation, and others, face many constraints such as R&D limitation, few positive outcomes, low acceptance from the general public, concerns regarding quality, authenticity and reliability and again, high prices implying an immobilised commercialization.

For this reason, the potential market for these non-medical applications should be exploited to identify the appropriate sectors to develop these biosensor devices in a way that end users would accept its use and incorporation to the market[170,172].

Moreover, there is not a standardized way to develop biosensors for multiple purposes. Thus, each different technology and approach needs to be studied and verified to try to validate it to the market and assess its correct incorporation and functionality.

3.2 Comparison between living biosensors and physical-chemical biosensors

The term sensor is widely extended and understood in our society, and it encompasses many different sensing strategies. Electrochemical sensors are the most common used and are based on the reaction between an electrode and an analyte. This electrochemical reaction then needs to be converted into an applicable quantitative or qualitative signal for its interpretation[173,174].

The main reactions occurring in these sensors for the information to be converted into measurable signals are changes in: i) current (amperometry), ii) potential or charge accumulation (potentiometric), iii) conductivity properties (conductometric), iv) impedance (impedimetric) and v) field-effect, which uses transistor technology to measure current as a result of a potentiometric effect at a gate electrode.
Electrochemical sensors can produce electronic outputs in digital signals for further analysis[175,152].

Electrochemical transducers are the most used transducers in the construction of innovative sensors including biosensors. They rank first in terms of their availability on the market and have already shown their true benefits. Electrochemical determination is superior to other measurement systems due to its quick and easy features.

3.2.1 Introduction to the term “bio-sensor”

A biosensor is a device that measures biological or chemical parameters by generating signals proportional to the concentration of an analyte in the reaction.

Biosensors are found in many different applications such as: disease monitoring, drug discovery, detection of pollutants, disease-causing micro-organisms and markers that are indicators of a disease in bodily fluids (blood, urine, saliva, sweat)[176,177].

The main components to create a biosensor device are the following:

- **Analyte**: A substance or molecule of interest which wants to be detected.

- **Bioreceptor**: A molecule that specifically recognises the analyte, also known as bioreceptor, e.g., enzymes, cells, aptamers, deoxyribonucleic acid (DNA) and antibodies.

- **Transducer**: An element that converts one form of signal into another. In a biosensor the role of the transducer is to convert the bio-recognition event into a measurable signal. In this case, the conversion of information is made through optical or electrical signals, which are proportional to the amount of analyte-bioreceptor. Moreover, this signalling can be generated in form of changes of pH, heat, charge etc.

- **Electronics**: This is the part of a biosensor that processes the transduced signal and prepares it for display. It consists of an electronic interface which can either amplify the signal generated or convert signals from analogue to digital. These signals are then quantified.

- **Display**: The display consists of a user interpretation system to present numbers or curves to be understood by the user. This part often consists of a combination of hardware and software that generates results of the biosensor in a user-friendly manner. The output signal on the display can be numeric, graphic, tabular or an image, depending on the requirements of the end user.

Although electrochemical sensors have many great advantages such as the robustness, the easy miniaturization, the detection limit, even with small volumes, the low-cost production as these are closely related to micro-electronic circuitry and the

ease implementation in relevant environments, these types of sensors encounter a relevant drawback such as the wide range of molecules being able to be detected[178]. When considering biological agents, this type of devices is very limited. For this reason, the combination of knowledge in bio- and electrochemistry, fused with data processing, offers the possibility of a new biosensor generation to undercome some of the current limitations.

3.2.1.1 Advantages of living technologies

In the recent years, many scientific studies have been published regarding the use of engineered organisms, such as bacteria or yeast, as biosensors for detection and diagnosis. This has been made by using already existing sensing mechanisms present in different living organisms. When identifying receptors in nature capable to detect specific molecules, these can be used to synthetically engineer a model organism to introduce a new function, in this case, the sensing capability of a specific target. This has extensively been done, verifying the ability of these modified cells to detect substances or compounds such as heavy metals, biomarkers secreted in the environment, quorum sensing molecules, among others. The use of these cellular biosensors is appealing for several reasons such as: i) can be used in a wide range of applications, ii) are ease and cheap to produce at industrial scale, iii) do not require additional lab or technological facilities[179].

However, although much research has been done, not a standard product for further commercialization has been designed, making difficult its technological transference to the market.

3.2.1.2 Key aspects to design a cell-based biosensor

A living biosensor should accomplish the following features to be interesting for the market and have a competitive position versus already existing devices[180]:

a) *Selectivity*: highly specificity for the analytes, stable under normal storage environment, and compatible over many assays.

b) *Accuracy*: the signal must be precise, accurate, reproducible, and linear over the desirable range.

c) *Biocompatibility*: the sensor should work satisfactorily *in vivo*.

d) *Durability*: currently available biosensors have a short lifespan and do not tolerate severe environmental conditions.

e) *Contamination*: avoid contact with the environment.

f) *Cost and size*: it must be small, cheap, portable, user friendly and capable of being used without the need of skilled personnel.

3.2.1.3 Two component biosensors

As mentioned before, there are different methods to detect biological compounds. In this chapter, we are focusing on the use of bioreceptors, i.e., well-studied proteins which can bind specifically to different molecules of interest.

The most typical examples for this type of bio-cognition elements are found for environmental pollutants such as heavy metals[181].

Microbial biosensors are naturally or engineered microorganisms which produce detectable signals in response to changes in the environment. Most of the engineered ones are based on bacterial designs which contain an inducible promoter or a receptor-promoter architecture which allows the detection of certain molecules of interest. This final promoter is usually coupled by a reporter gene which produces colorimetric, luminometric, fluorometric or output signals changes.

Many types of biosensors have been developed using various transcription factors and regulatory regions. In the most common scheme, a reporter gene is expressed from regulatory DNA regions (promoter).

From this basic architecture, a lot of research has been done to find sequences encoding proteins or responsive elements which specifically respond upon different molecules of interest, as well as natural operons existing in nature which allow certain species to live and adapt in specific environmental conditions.

Next-generation sequencing (NGS) technologies have generated an enormous amount of sequence information about cellular genomes and environmental metagenomes, from which we can select parts and modules[182]. Additionally, public websites which collect standard bio parts such as the Registry of Standard Biological Parts (http://parts.igem.org) are great repositories to find promoters, operators, regulators, pathways, etc.

Moreover, gene synthesis and genome editing technologies have largely evolve, making easier the construction of tailor-made designs for the detection of specific molecules or the synthesis of biochemicals by engineered cells.

When designing a biosensor, multiple genetic architectures can be considered. Among them, the two-component systems are appealing because of their simplicity. Two-component systems consist of a sensor protein (input domain) and a response regulator (receiver domain). The first domain responds to the presence of a molecule of interest, which subsequently activates the receiver domain[183] . Using two-component systems, cells can detect a wide range of environmental signals, including light, oxygen, pH, temperature, heavy metals, and organic contaminants.

Figure 3.1 shows a schematic representation of a two-component system which activates the receiver domain when dimerizing with a specific molecule of interest.

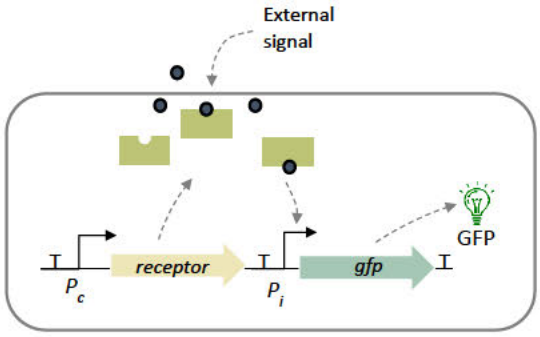


Figure 3.1 General architecture for an inducible two-component biosensor. When a specific receptor binds to the molecule of interest *i*, the inducible promoter (P_i) responds through the production of a fluorescence protein.

For the reporting part, cell-based biosensors must convert the sensing cascade into a physiochemical signal. The main reporter options are colour changes, bioluminescence, and fluorescence, among others. As the transcription of these reporter genes is upregulated by a specific promoter or operon responding to the molecule of interest, reporter levels are proportional to the signal being sensed.

Fluorescence is widely used as a reporter system because of its high stability and ease of measurement, without a requirement for exogenous substrates. However, fluorescent reporter proteins have some drawbacks, including the relatively long time required for expression of stable fluorescent proteins and the need to distinguish auto-fluorescence or background fluorescence from the real fluorescent signal emitted by cells during measurement[184,185].

3.2.1.4 Current limitations of whole-cell biosensors

Most existing whole-cell biosensors are appropriate when working in a controlled environment and using complex infrastructures for culturing or measuring, as the bioproduct production or the colour intensity are parameters which need additional hardware to be interpreted.

However, the development of successful commercial cell-based biosensors has been slow due to several challenges regarding the design and the ability to sense targets relevant concentrations. Thus, the development of rational methods to tune biosensor performance and the increased number of available parts has led to renewed interest in biosensors as its construction and optimisation has become much quicker.

Additionally, biosensors face challenges in acceptance arising from biosecurity fears, and concerns over the duration and response stability, robustness[186] and reliability of the sensors and the methods for determining results[187]. These challenges are mainly because tested samples usually contain many pollutants or naturally occurring molecules which mask the signal coming from the molecule of interest, or which can also be toxic for cells used to perform the detection. Finally, response from whole-cell sensors employed for extended periods of time can become unstable, obtaining wrong results[188].

Finally, further advancements in miniaturization and portability of signal detection systems for real-time detection are needed, enabling point of care measurement. Moreover, microbial strains permitting simultaneous multi-analyte detection with the proper specificity is another great challenge [189].

3.2.1.5 Regulatory framework

In biosensor commercialization, key factors determining its commercialization are simpler sample pre-treatment, bioreceptor stability, multi-detecting properties, miniaturization, shorter testing time, wireless availability, and affordability. Common features of commercialized biosensors are their simple construction, smaller sizes, and ideal qualities for POC applications.

A fundamental factor that determines the future of a biosensor is its safety in human health, which means that only those biosensors and related technologies with minimum or no human health impact will have their commercialization future in the coming years. In our case, Directive 2009/41/EC regulates the contained use of genetically modified organisms (GMOs).

The term "contained use" covers any activity involving genetically modified micro-organisms (including viruses, viroid, animal and plant cells in culture) that is carried out under containment to limit contact of these organisms with the environment[190].

Besides assessments and testing methods, a harmonized standardization of components and parts can accelerate effective commercialization of synthetic biology-based biosensors. At the current stage of lifecycle, a flexible form of regulation for synthetic biology-based biosensors can provide a good solution for its effective and success growth. Most of the technologies including life sciences thrive on innovation and generation of intellectual property (IP) in the process of innovating. Protection of intellectual property right (IPR) serves as an incentive for both the creator and the society.

However, as the biosensing circuits would be designed using well characterised standard parts, the question of ownership, availability of these parts and mode of protection needs to be resolved. Increase in significant investments and launch of commercial synthetic biology derived biosensors products will depend on the addressal of the said issues of governance[191].

3.3 Biosensor architecture for the detection of chemical compounds

As we have already pointed in the previous section, synthetic biology and cellular sensors are appealing to sense a variety of targets. The motivation for this chapter is to overcome some of the current limitations, such as the difficulty to use this cell-based sensors out of the lab and to measure the output produced by these systems without the need of infrastructure or complex machinery. For it, we seek to establish the basis for a new architecture of cellular biosensors which can work out of the lab and can offer a visual quantification response without the need of complex equipment for the readout.

As previously seen, most living biosensors are whole-cell biosensors which integrate all the genetic circuitry in one cell, which is capable to perform the detection.
Although this can be a good approach, here we explore the use of different cell types to perform the detection and the quantification, together as the space, to easily quantify what our engineered cells are detecting. In the second chapter of this thesis, we have demonstrated that cells can perfectly grow and communicate on paper surfaces. Moreover, we have combined computation together with spatial distribution and distributed computation to achieve more simple designs to undertake more complex functions.

Wrapping up all these concepts, we have designed this new architecture to map any potential biosensor device used for the detection of chemical compounds.

These biosensors will be implemented using E. coli as a model organism and devices will contain two distinguishable parts: the detection/transduction module and the readout module. Those will be optimal for rapid *in situ* detection without the need of lab facilities or complementary equipment. It is worth mentioning that this biosensor

devices are expected to be easy to use and cheaper than other commercial systems. However, it should also be pointed that they will contain living cells and hence, will have an expiry time to use.

3.3.1 Creation of a detection/transduction module and a readout module

The transduction module is composed by an engineered cell which includes the elements needed to undertake the detection (i.e., receptors, inducible promoters, transcription factors etc) coupled with the production of a diffusible molecule. In this case, rather than expressing a reporter gene, we want to incorporate the expression of the well-known acyl homoserine lactone (AHL), which would be produced based on how much input is being detected. Then, when the target of interest couples to the receptor protein, this triggers the expression of the gene encoding the enzyme LuxI. This enzyme catalyses the secretion of AHL molecules.

The second module, i.e., readout module consists of the already explained CR cells from the second chapter, which contain the LuxR/pLux machinery to detect this diffusible molecule and produce GFP as the output signal.

The disaggregation of the detection and transduction from the readout enables to reuse this last module in each biosensor device, only replacing the cells in the first module which contain the specific architecture to detect a molecule of interest.
Figure 3.2 presents a schematic representation of the genetic circuitry for each different cell type involved.

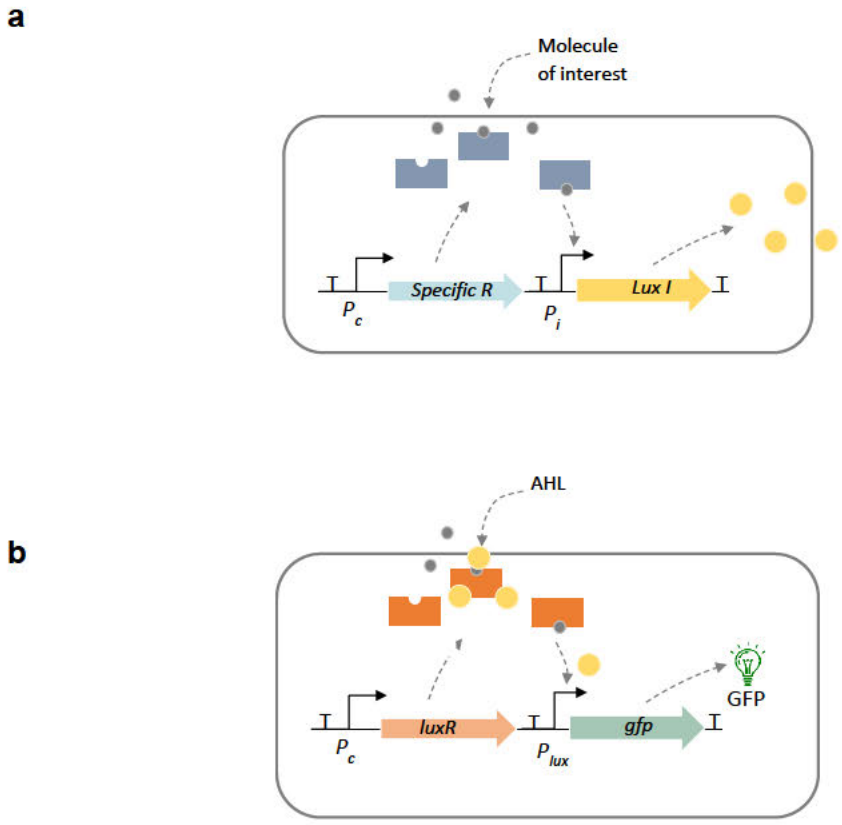


Figure 3.2. Genetic architecture for each biosensor module. a. Inducible construct for the transduction module. Upon the presence of a certain molecule of interest, it starts secreting AHL, our carrying signal or diffusible molecule. **b.** Reporter module. This is the common modules for all the biosensors, which reports de AHL concentration and produces GFP accordingly.

It should be pointed that in Fig.3.2, a two-components system is being displayed. Although it is true that not all chemical compounds can be detected through this type of systems, the idea is to design this first module according to each specific target of interest and adopt it to the architecture which will be explained below.

3.3.2 2D printed biosensors which can quantify abundance depending on the diffusion pattern

The idea of using these two cell types is interesting when being coupled with the 2D surface and the diffusion pattern of the secreted molecules, i.e., AHL. On the one hand, the transduction module will grow at one end of a piece of paper located over a

surface of Lysogenic Broth-Agar. At a certain tested distance, different equidistant dots will be printed along the paper with the second readout module, having cells capable to detect AHL molecules and produce GFP, i.e., CR cells.

Then, depending on the concentration of each target molecule, the total amount of AHL produced by the transduction cells will be different, and consequently, it will be translated into different diffusion gradients.
Depending on these diffusion gradients, it will be possible to estimate the initial concentration, as a different number of reporting dots will be turned ON.
The sample to be analysed is thought to be dropped over transducer cells, which will initiate the response.

This theoretical scheme (Fig.3.3) illustrates the system logics and the potential responses to quantify the elements being detected. As seen in the image, there is a non-linear relationship between the amount of detectable signal and consequently, of AHL being diffused to the media, and the GFP response based on the amount of AHL molecules being detected. For this reason, this non-linearity requires a system calibration to associate distances, i.e., diffusion patterns, with the concentration of the signal being detected.

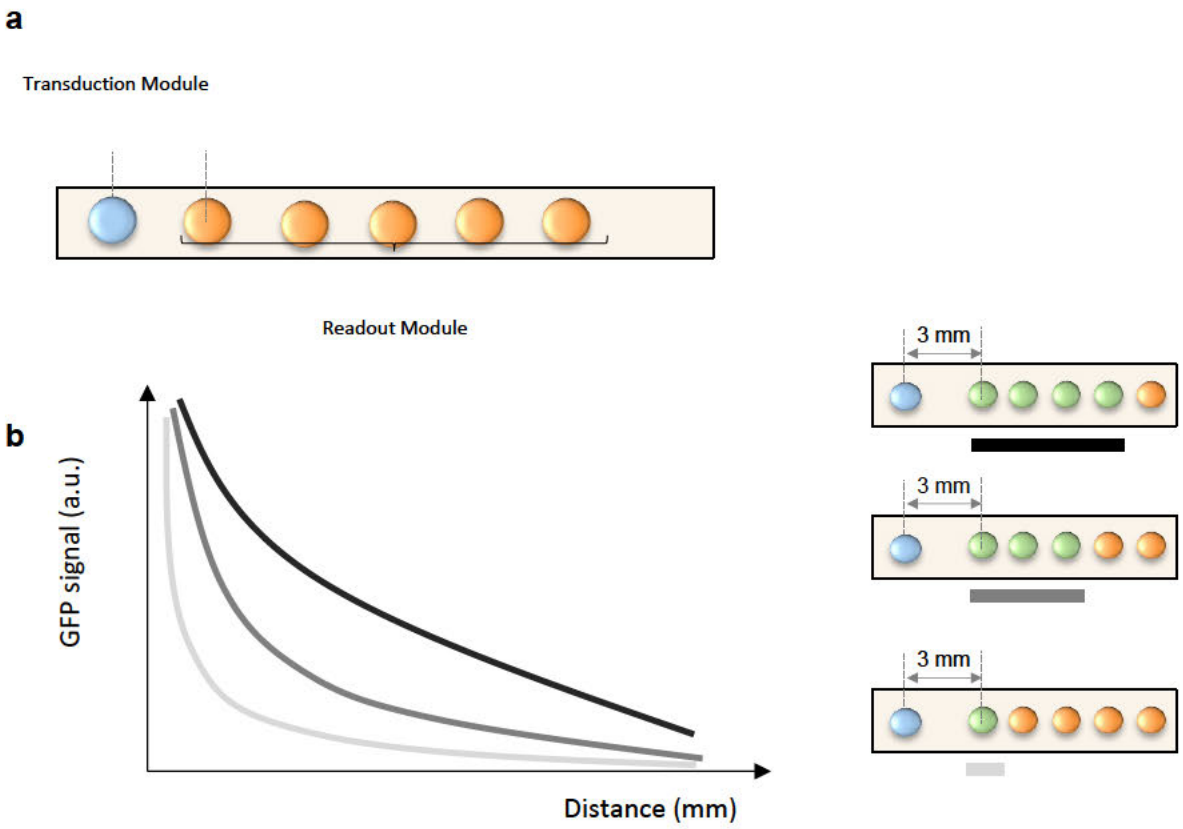


Figure 3.3. Diffusion pattern in 2D surfaces as a quantification method. a. This image illustrates the general architecture on top. **b.** Depending on the amount of molecule being detected, different AHL amounts will be secreted and thus, different diffusion patterns will be achieved. This diffusion patterns directly correlate with the number of reporter cells being turned ON, i.e., green dots.

The key aspect of having these two engineered cellular designs is the way these are placed in the space to avoid the use of any type of machine to perform the readout.

3.3.3 3D prototypes to display how cellular elements are arranged in a device

As mentioned before, these biosensors are thought to meet market needs. Thus, an important point of this work was to identify the key elements which would configure the device.
These devices include the following elements:

- Chambers: two different chambers for each engineered cell type. These chambers contain a layer of Lysogenic Broth-Agar to give nutrients to all cell types and a piece of paper strip on top, containing the engineered cell types.

- Lid: devices must be covered, and we were considering a methacrylate lid to enable the output visualization and the cell growth. All cells are tagged with a constitutive RFP. Then, if a cell type is not growing it will be detected by the absence of red colour.

- Ruler: each device will be characterized with each specific diffusion profile and a ruler will indicate the target concentration present in the sample depending on the output distance.

The figure 3.4 depicts the biosensor architecture with each module.

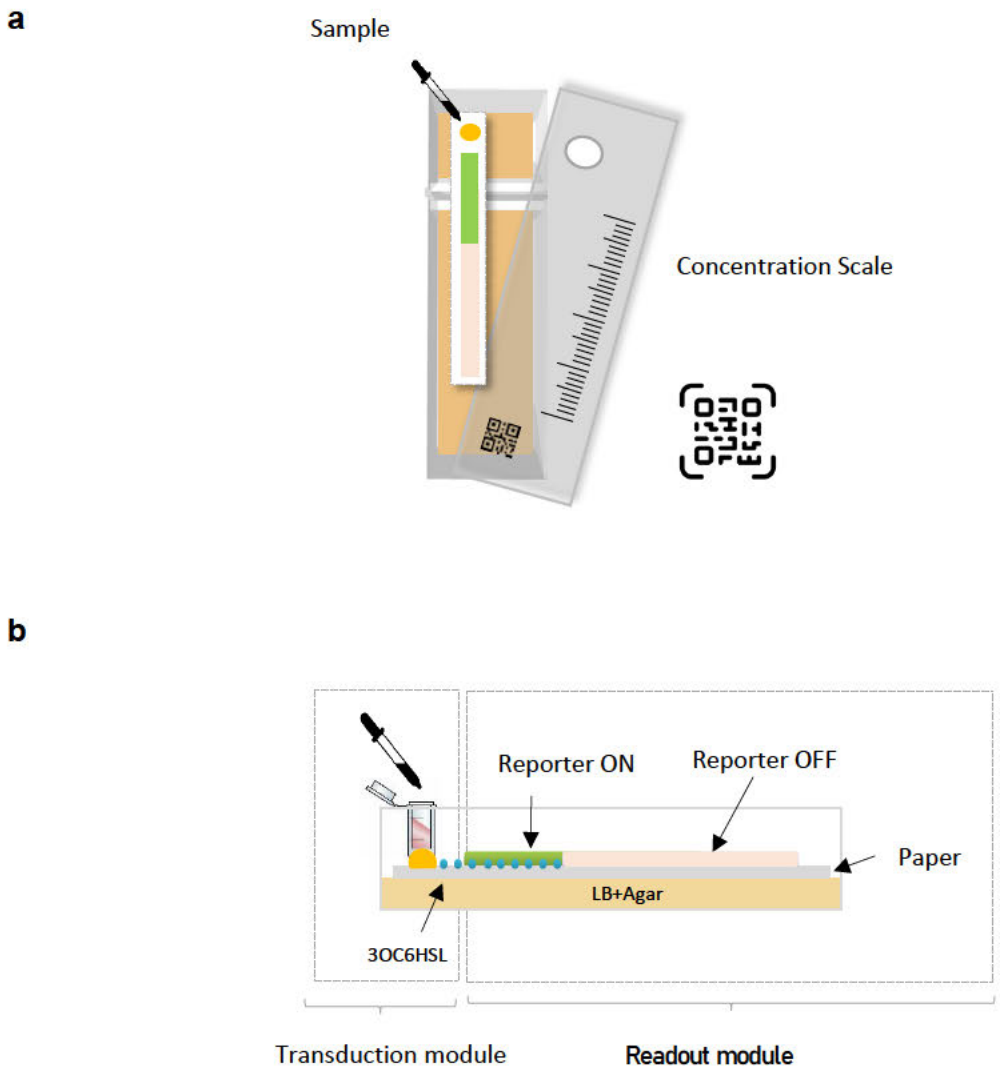


Figure 3.4. Prototype design. a. On the outside, the idea is to display the output, i.e., quantification through the diffusion pattern, next to a ruler which would be first calibrated depending on what is being detected. It would also contain a lid with a sterilized spot to place the sample. Additionally, there is a QR code to be scanned to keep a record of all the measurements. **b.** On the inside, the different layers can be seen, having the substrate (LB Agar with the paper strip), both transduction and reporter chambers and cells being deposited in each compartment. AHL molecules are represented as blue circles, mimicking the diffusion through the reporter chamber.

Regarding additional functionalities, the idea of having a QR code in the lid is for two main purposes. On the one hand, this QR was thought to be scanned to open an app so the user could add results, thus enabling to keep the historical data of what is being analysed. On the other hand, we were thinking on offering image recognition from this

QR scan, offering an autonomous image interpretation, so the user would not need to look for the result.

From this first concept, different prototypes were then designed with Sketchup software to then 3D print them, looking for the best configuration to obtain a functional prototype.

In the next section, specific examples are tested to check the viability of the proposed biosensor architecture for specific chemical compounds, in this case, for a mercury biosensor.

3.4 Prototyping a mercury biosensor based on this new architecture

3.4.1 Defining the problem

3.4.1.1 Why does mercury need to be detected and where?

Heavy metals are naturally occurring elements that have gained ecological and global public health concern because of its increasing exposure and accumulation. This rise comes from anthropogenic activities such as mining, smelting, industrial production, i.e., plastic, textiles or in microelectronics, and due to domestic and agricultural use. However, this accumulation can also come from metal corrosion, atmospheric deposition, soil erosion of metal ions and volcanic eruptions, among other natural phenomena[192]. Thus, inappropriate concentrations of these metals have been found in soil and water, indirectly affecting food security, and being incorporated in the food chain.

Heavy metals are systemic toxicants and are classified as “known” or “probable” human carcinogens, even at very low concentrations. These heavy metals are the main toxicity-generating elements for living, identified by the World Health Organization (WHO) since they are non-degradable and tend to bioaccumulate.

Worldwide, it is estimated that 20 million hectares of arable land are irrigated with wastewater. Moreover, in many areas in Asia and Africa, this water is used for irrigating half of the vegetable supplies. This, in turn, affects the public that is consuming this

type of food, suffering some of the above-mentioned causes or accumulating undesirable toxicants in the body[193]. Although large improvements have been done, detection of these metals still relays on spectroscopic techniques, which are highly sensitive but also expensive and require special facilities and qualified operators to perform it. In addition, there is not yet an effective way for its bioremediation[194].

To mitigate this massive contamination, there is the increasing need of finding sensitive, rapid, reliable, and cost-effective tools to detect these compounds, which can also assess parameters such as toxicity, bioavailability, and genotoxicity[195].

On this first example, mercury detection is carried thanks to a mercury-resistant bacteria which was reported to contain genetic mechanisms to capture ionic mercury from the environment. One of the best-studied mechanisms to do so is the Mer operon, which is a positively inducible operon and is responsible for the detoxification of mercury. MerR, a metal-specific activator repressor of the operon encodes the MerT, MerP, and MerA structural genes. These three components (transport, catalysis, and regulation) are the central functions of mercury resistance operon[196].

When Hg^{2+} is present, it binds to MerR provoking an allosteric change of PmerT, leading to an underwinding of the operator DNA and an improved access of RNA polymerase to the transcriptional start site and expression of downstream genes.

First, MerP detects Hg^{2+} in the extracellular space; afterwards, MerT (a membrane protein able to recognise MerP- Hg complex) transfers Hg^{2+} into cytoplasm and binds it to MerA, which reduces Hg^{2+} to Hg^{0}, that can cross the cytoplasmic membrane by diffusion and finally leaves the aquatic environment to the atmosphere[197]. These processes are represented in figure 3.5.

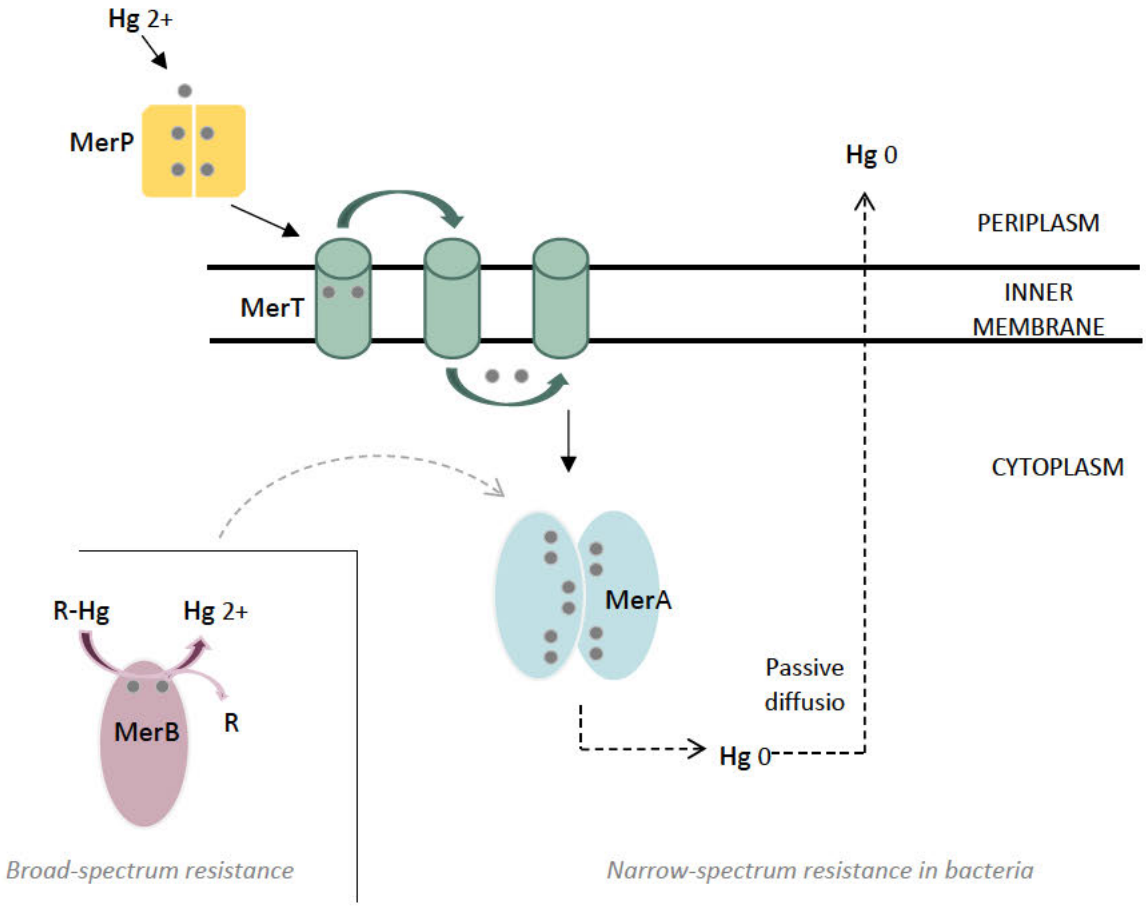


Figure 3.5. Mercury Operon. This scheme depicts the main regulatory elements involved in the mercury uptake and bioremediation.

3.4.2 Mercury sensor characterization

Not surprisingly, in literature, many articles have described the use of whole-cell biosensors to detect mercury concentrations using elements from its operon. In our case, we first constructed a two-components whole-cell mercury biosensor based on previous literature[71,195,198–200] to have a proper biosensor characterization to then adapt it to the new architecture described before, using this two modules to undertake the detection and analyte quantification.

This two-components biosensor was made through the construction of an engineered plasmid which contains the receptor protein merR responsible to bind to mercury ions together with the inducible mercury promoter (Pmer), which is subsequently activated

and can lead to the further expression of the downstream genes. This engineered construct was transformed into E coli DH5α strain for its characterization.

Moreover, we made this the MerR protein inducible (i.e., with an arabinose inducible promoter) to explore the role of the amount of receptor, in terms of biosensor sensitivity, as this feature is dependent of the receptor abundance[184], as well as the effects of having an excessive receptor expression, which implies a lower cell surveillance[195]. We then added a GPF downstream the Pmer promoter to correlate the mercury concentration with the amount of fluorescence being emitted.

Figure 3.6a shows a schematic representation of genes involved in mercury ions detection.

To characterize this engineered construct, the system was tested upon nine different arabinose concentrations, i.e.,10^{-3} to 10^{-9} M mixed with LB media to assess cell viability depending on MerR expression.

Afterwards, the system was characterized using three of these nine different arabinose concentrations based on the results obtained from the previous experiment, i.e., using 10^{-6} M, 10^{-7} M and no arabinose, as higher arabinose concentrations implied lower growth rates.

From these three arabinose concentrations, we prepared a serial dilution of $HgCl_2$, i.e., from 10^{-5} M to 10^{-10} M to test the actual system response upon the presence of mercury. Thus, our LB media had a mixture of arabinose and a dilution of $HgCl_2$.
Thus, we could finally determine the right receptor induction and the operating range of this biosensor.

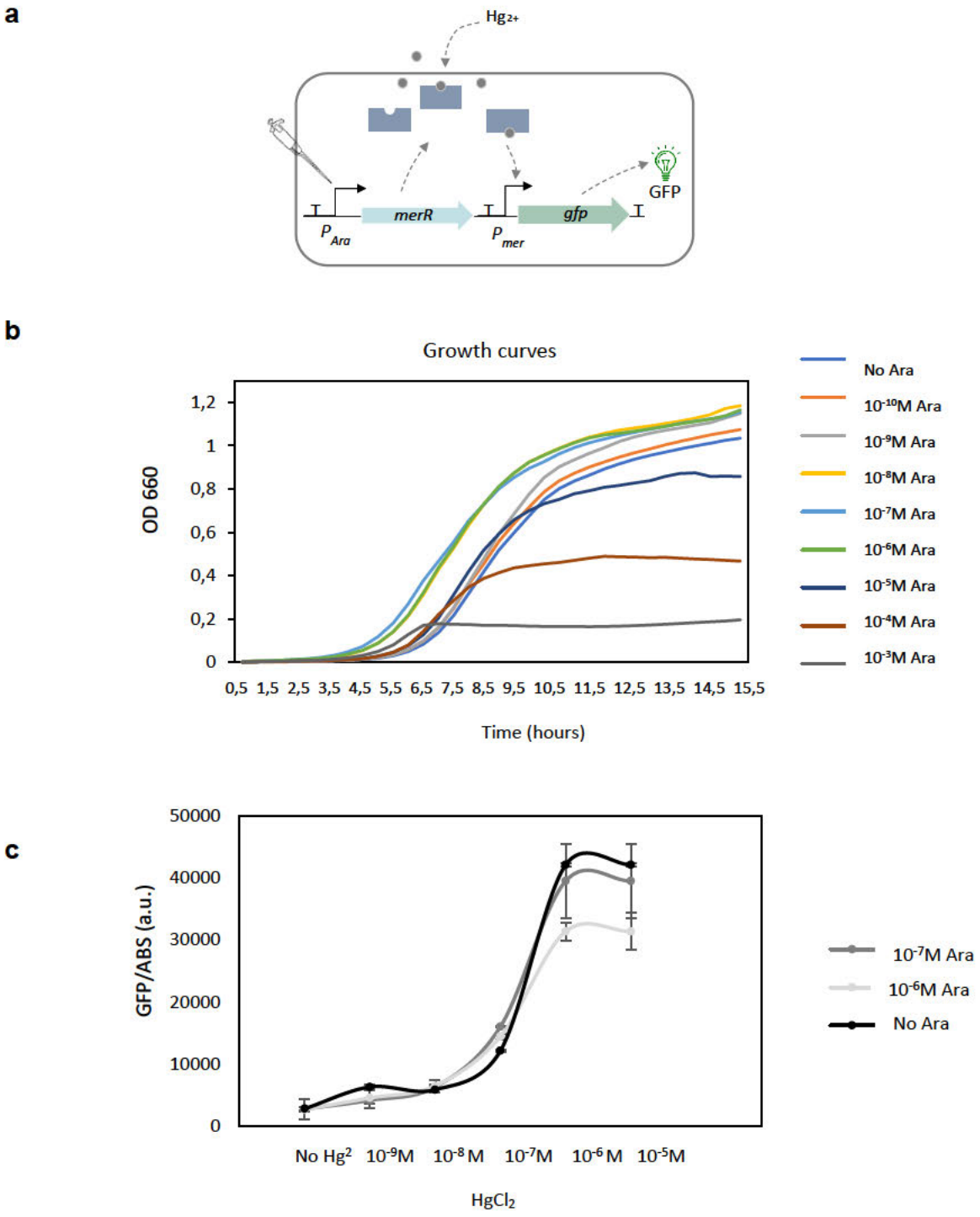


Figure 3.6. Two components mercury biosensor characterization. a. Genetic architecture of a mercury responsive construct upon the arabinose external induction. **b.** Cell viability depending on different arabinose inductions. MerR was known for being toxic at high expression levels. For this reason, an arabinose promoter was used to assess cell viability based on OD660 measures. Each colour line corresponds to a different arabinose concentration for growth assessment. **c.** System characterization with different arabinose concentrations and upon a serial dilution of $HgCl_2$, from 10^{-5} M to 10^{-9}M. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.

As seen in the Fig.3.6b, this biosensor has different behaviours depending on the arabinose concentration, which determines MerR abundance. However, it was seen that with the small leakiness of the arabinose promoter, it is enough to transcribe the receptor to detect mercury ions and achieve a good response upon different mercury concentrations. Moreover, when using higher arabinose concentrations, the bacterial construct was not growing properly. For this reason, serial dilutions with $HgCl_2$ were not carried with these higher arabinose concentrations, i.e., 10^{-3} to 10^{-5} M.

Interestingly, previous groups detailed that the weaker the promoter (that is, the lower the MerR receptor concentration), the more sensitive and higher the dynamic range of the sensor.

In Fig.3.6c, the operation range on this biosensor is analysed, ranging from 10^{-8}M to 10^{-5}M, as 10^{-4}M is toxic for cell as these did not grow. As it was previously stated in other studies, it could be seen that the lower, or even inexistence of arabinose induction, the greater the response upon mercury presence. Thus, it was determined that no arabinose induction was needed to test the performance of this biosensor as the leakiness of the arabinose promoter was enough to display an appropriate response.

This engineered biosensor was also tested with water from the Ebro River provided by ETAP (Estació de Tractament d'Aigua Potable) in Tarragona, which regularly testes the water to check heavy metal abundances with mass spectrometers.

To perform this analysis, we prepared the regular Lysogen Broth (LB) medium for bacterial growth, i.e., prepared with LB powder, containing Yeast Extract, Bactotryptone and Salt, mixed with distilled water to dilute this powder, but in this case, the water used was the one to be analysed from the Ebro River, rather than distilled water (Fig 3.7a).
Moreover, no arabinose was used for MerR expression based on the great performance of the biosensor, having similar results than using 10^{-7}M arabinose induction (see Fig. 3.6c). Regular LB media was used as a control measure to assess the biosensor leakiness. Results can be seen in Figure 3.7b. Biosensor response was measured based on the GFP expression.

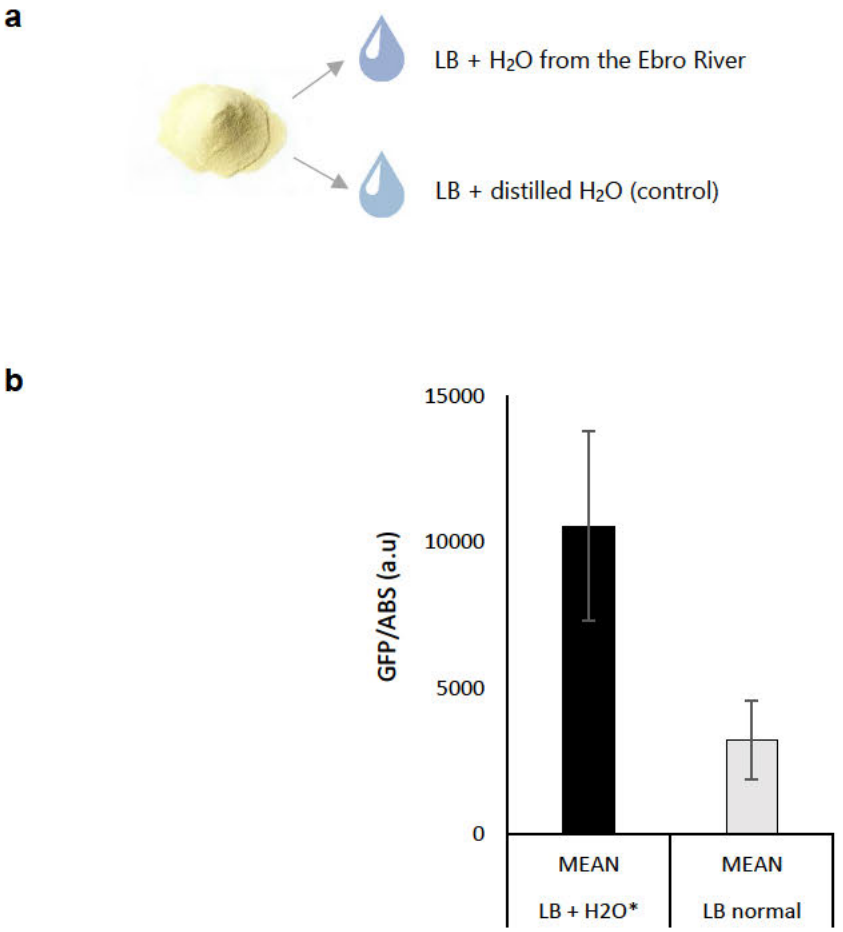


Figure 3.7. Testing the biosensor with water from the Ebro River. a. Experimental setup for the sample preparation and its corresponding control. **b.** Four independent experiments were performed to measure the biosensor response using normal LB as our control measure and LB prepared with water from the Ebro River as our measured sample. Error bars are the standard deviation (SD) of four independent experiments. Data are presented as mean values +/- SD.

As seen in the Fig.3.7b, although there were some variabilities between samples, a higher GFP expression was clearly observed in the media prepared with the water to be analysed. Based on the experiments performed previously (Fig.3.6c), we could determine a mercury concentration around $5 \cdot 10^{-7}$M and ETAP company reconfirmed our results with their Mass spectrometry analysis. The estimated mercury concentration was 0.135ppm. It is worth mentioning that, by law, the EPA has established a limit of 2 parts per billion (ppb) of allowable mercury of drinking water.

The Food and Drug Administration (FDA) has set a maximum permissible level of 1 part of methylmercury in a million parts of seafood (1 ppm)[201].

These experiments were carried to characterize the mercury construct and are consistent based on previous works[71,195,198–200,202]. Moreover, we could determine great sensitivity and great potential to test the architecture explored in this chapter. Thus, to verify its adaptability, we built the modules we presented before, i.e., a transduction module and a readout module, in this case, for the particular example of the mercury biosensor.

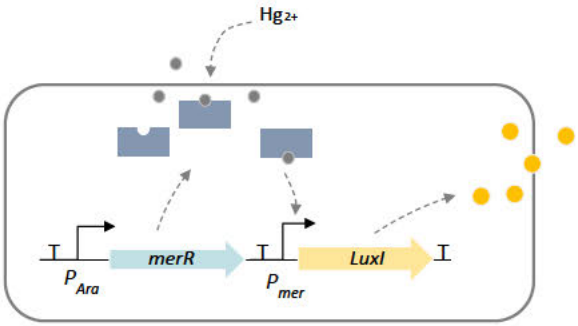
3.4.3 Design of the engineered modules for the mercury biosensor

3.4.3.1 Transducer module and Detection module

From the theoretical design previously explained, we have created a very simple paper-based biosensor prototype responding to mercury. This device is based on the minimal architecture shown in figure 3.8 composed by an inducible mercury transduction module that secretes AHL in the presence of mercury ions (Fig, 3.8a), combined with a reporter cell (CR) expressing GFP in response to AHL molecules as our readout module (Fig 3.8b).

CR cells conforming the readout module are being distributed in dots along the paper strip. Then, depending on the mercury concentration, different amounts of AHL are produced, i.e., higher AHL production implies larger AHL diffusion, and thus, different number of dots will be turned ON, i.e., expressing GFP accordingly.

a



b

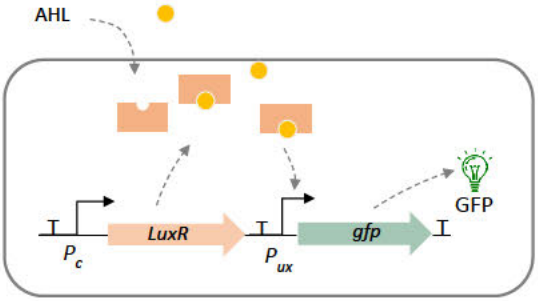


Figure 3.8. Genetic architecture for the mercury prototype. a. Specific transduction module for mercury detection. This engineered construct produces AHL upon the presence of mercury ions. **b.** General readout module for all biosensor devices. This engineered construct produces GFP depending on the AHL concentration. This construct is the same as CR cells from the second chapter.

3.4.4 3D prototype assembly

As mentioned before in the prototype design section, different moulds were printed and tested to look for the best design.

In the figure below (Fig.3.9), prototypes are depicted with all its compartments. This small mould enables to fill compartments with different mixture combination, for instance, filling our Lysogen Agar media to solidify before adding the sample to analyse.

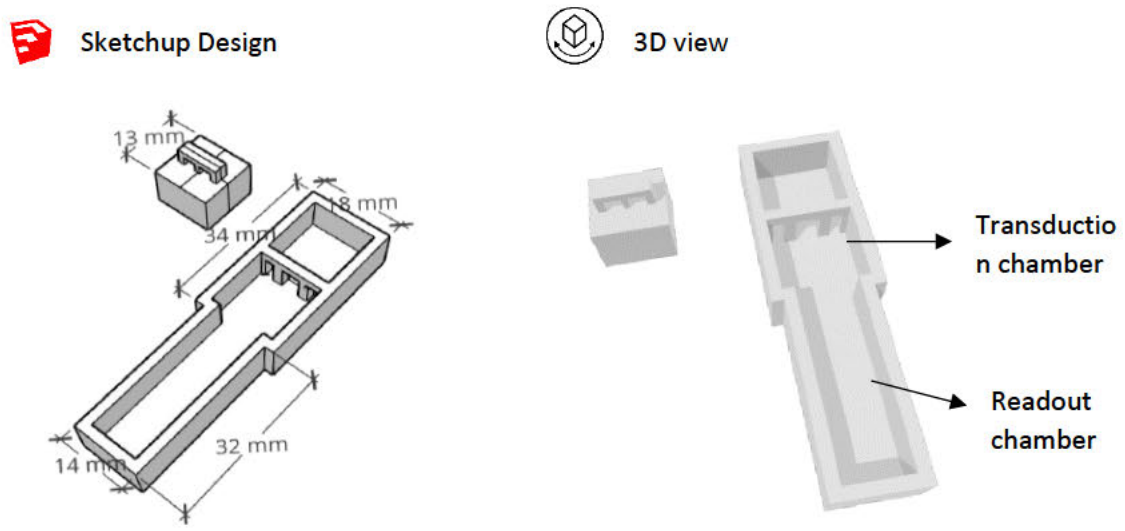


Figure 3.9. 3D prototype printing. This image shows the schematic design of the prototype to test the proposed architecture. This mould was filled with LB Agar and each cell type.

As a first validation, the transduction compartment was filled with serial dilutions of mercury concentrations mixed with LB Agar. Although the final device pretends to add the sample in on top of the first module or in a first compartment directly from liquid phase, this approach was carried as the amount of liquid was unknown and we wanted to test the device performance in experimental conditions. The rest of the prototype (the readout compartment) was filled with LB agar alone. On top of this surface, a paper strip was placed, and different drops were printed on top of it, one for the transducer and ten more for the reporter module.

After filling these prototypes, moulds were covered to avoid any contaminations and were stored at 37°C for an overnight.

Based on this setup, Figure 3.10 depicts the final device configuration.

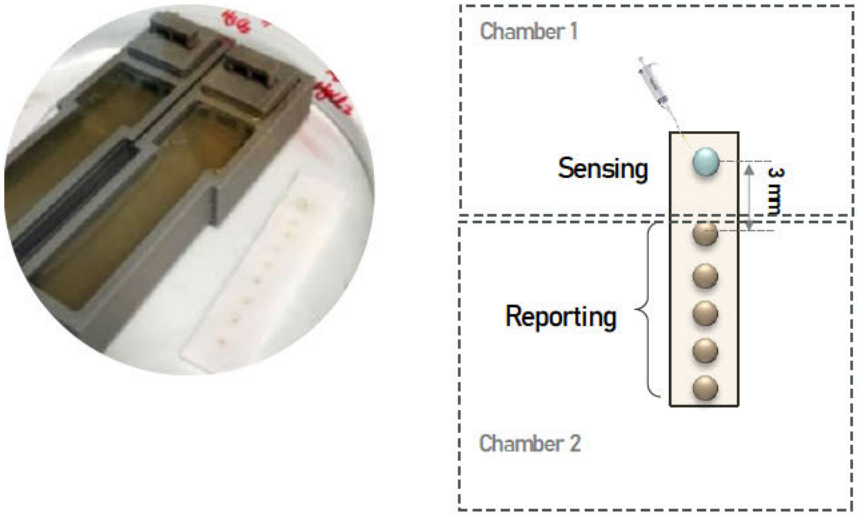


Figure 3.10. Prototype assembly. A. Schematic representation of the system configuration. B. Picture with the final assembly after being inoculated at 37° for an ON.

3.4.5 Biosensor characterization and performance assessment

As seen in the picture above, the way reporter cells are distributed is by adding multiple equidistant dots at a predefined same distance to allow visual quantification. In consequence, the number of dots expressing GFP (green dots) correlates with the mercury concentration and can be estimated by naked eye or under a small blue LED transilluminator.

Experimental results are shown in figure 3.11. Figure 3.11a shows the correlation between mercury concentration and number of green dots observed. Figure 3.11b shows photographs of the visual readout at different mercury concentrations. Results demonstrate a responsive range between 0.013 and 2.71 ppm. Based on regulation standards, it is established that the allowed amount of mercury in most commercialized products is between 0.1 and 1 ppm. Thus, this prototype could be optimal for a fast and easy product screening to ensure product safety.

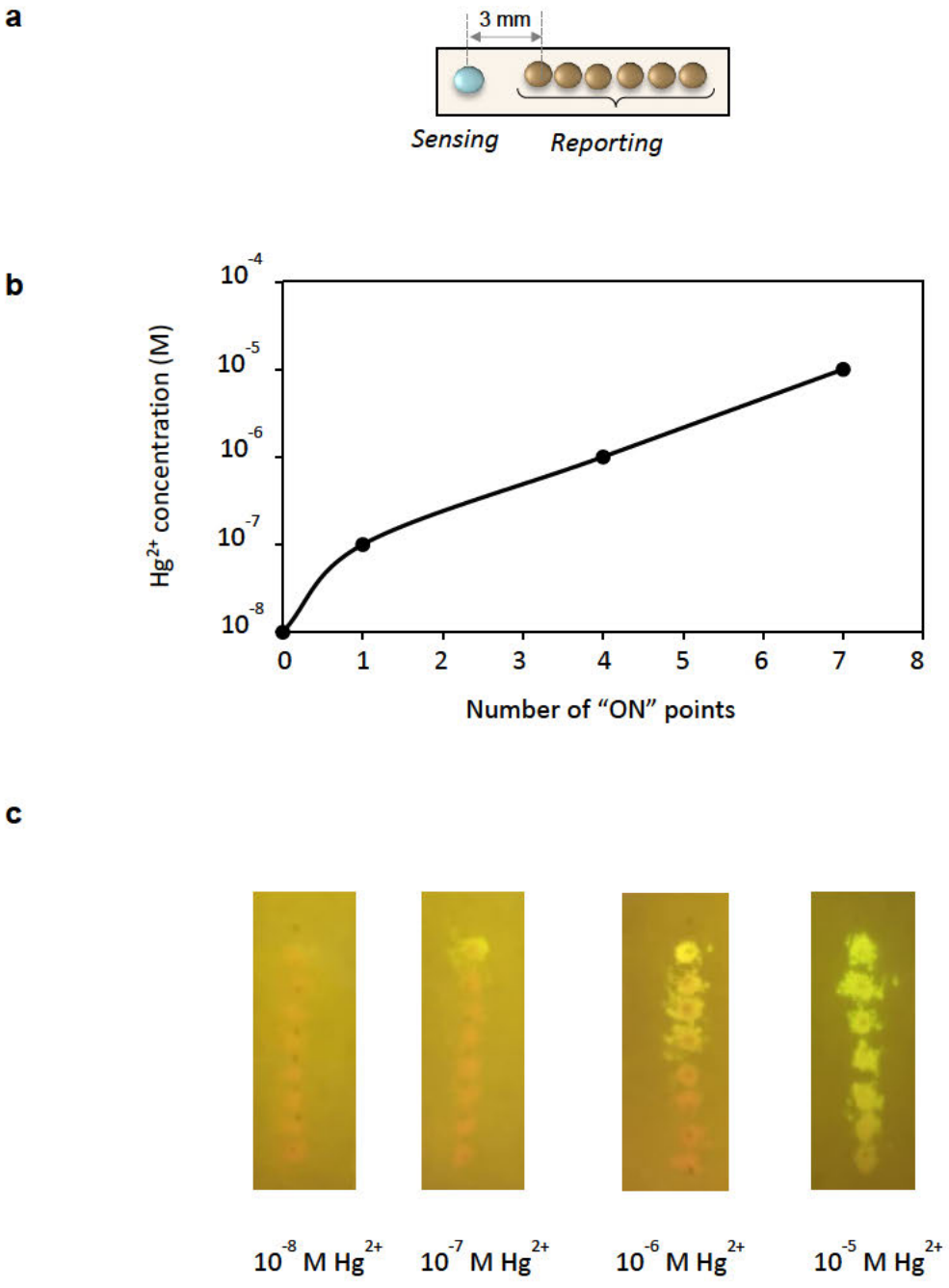


Figure 3.11. Mercury biosensor prototype. a. Schematic representation of the different cells used for the biosensor assembly and characterization. In this case, S_4 cells are engineered cells which respond to different mercury concentrations and produce AHL molecules. The production of AHL is dependent on the mercury concentration, thus, the more mercury in the media, the more AHL signal is released. This signal sent is then detected by CR cells. **b.** Correlation between the CR points turned "ON" and the mercury concentration is displayed. As seen in the pictures, a visual quantification can be obtained by checking the number of CR cells "ON", i.e., expressing GFP. Three independent experiments were performed from each mercury concentration and results are consistent.

This visual estimation was then quantified to have the proper quantification of GFP expression for each mercury concentration. The figure below (Fig. 3.12) correlates GFP, normalized with the corresponding RFP (used to estimate cell population), as it was done in the second chapter of this thesis, versus each mercury concentration. These results were obtained by scanning all paper strips from the prototype after being inoculated at 37° overnight.

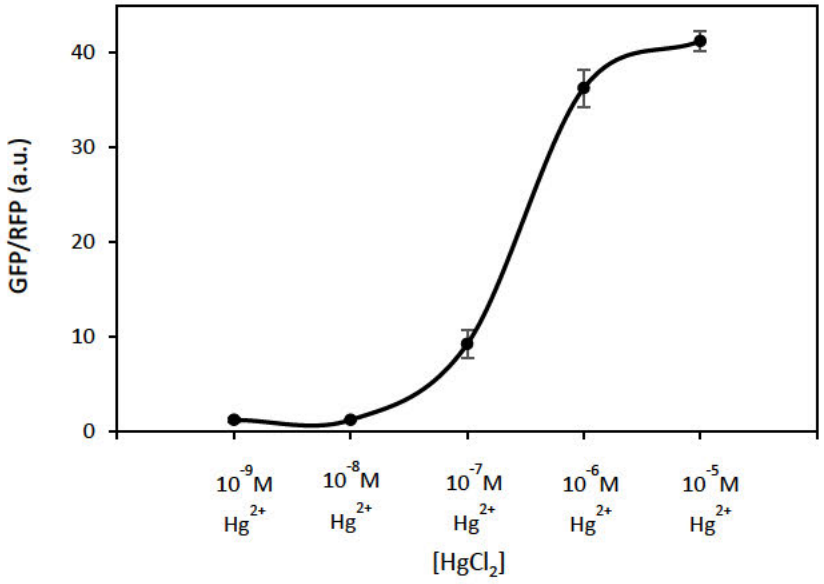


Figure 3.12. Prototype quantification. Each paper strip was scanned for a more accurate response quantification. This chart represents the GFP expresion for each mercury concentration, noramlized by the RFP as it is constitutively expressed. As it can be seen, activation ranges correlate with the number of turned on dots. Error bars are the standard deviation (SD) of three independent experiments. Data are presented as mean values +/- SD.The average fold change of the system is 35x.

As seen in Fig.3.12, similar results were obtained from the first whole-cell mercury biosensor characterization in liquid phase and this second approach using a new architecture design. The great advantage of this method is the ability to quantify without using any type of specific machinery and using signal's diffusion gradients to determine the abundance of mercury in the sample.

Nevertheless, it is true that to make this device work out of the lab, the analysed sample would need to be added directly and not in a mixture of LB Agar as it was done in this characterization.

3.4.6 Future work

The final aim of this mercury biosensor was to be able to add additional heavy metal sensors to have a larger screening of potential pollutants on samples of interest, as a point-of-care, easy to use device. We have checked potential applications for the use of this type of devices. For instance, in recycled paper, it is mandatory to check lead, cadmium, mercury and hexavalent chromium concentrations to be used for packaging purposes[203].

A schematic theoretical representation of these integrated devices for multiple metal detection is depictured above (Fig. 3.13).

To do so, we checked already existing sensing metal-responsive architectures to adapt them to the system described above[204].

- Lead: we would use parts of the chromosomal lead operon of *Cupriavidus metallidurans* (*Ralstonia metallidurans*). The promoter PbrA is regulated by the repressor pbrR. The PbrR protein mediates Pb2+-inducible transcription. PbrR belongs to the MerR family, which are metal-sensing regulatory proteins[205].

- Chromium: its detection consists of the chrB repressor and the chromate specific promoter chrP. The promoter chrP is regulated by the repressor, which binds Cr-ions. This repressor is originally from from Ochrobactrum triti ci5bvl1.

- Cadmium: this sensor can be built by combining operator binding sites for two metal sensor repressor proteins, ArsR and CzrA (cadA promoter)[206,207]. They both work as repressors and can bind to the DNA when they are bound to various metals. ArsR can bind to cadmium, silver, copper, and arsenic whereas CzrA can bind to zinc, cobalt, nickel, and cadmium. Cadmium can bind to both proteins and using a combinatorial approach enables to filter out any metals

other than cadmium. When cadmium is present both proteins are unbound from this AND gate and the promoter region controlled by the repressors becomes free to drive the expression of downstream genes such as GFP.

CadA system works as an efflux system to take the metals out in the cell. It is normally repressed by the CzrA (formely YozA) repressor protein.

When metals such as copper, zinc and arsenic are present inside the cell, they can bind to CzrA and derepress the promoter expressing CadA. Hence metals are taken out by the efflux system.

By utilising the promoter from the CadA system, it is possible to create a metal sensor that responds to at least these three metals.

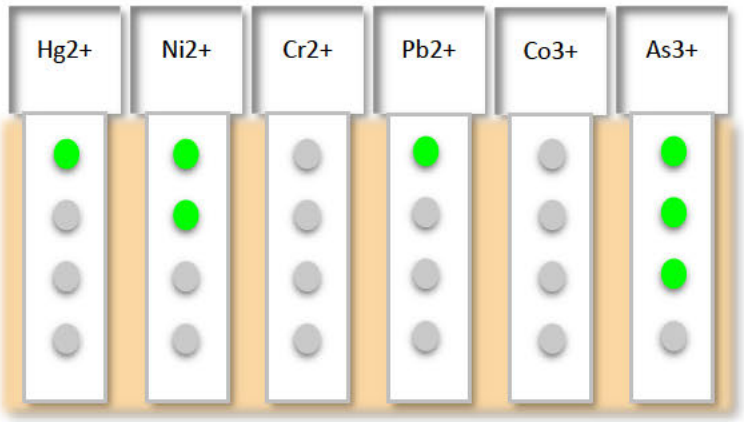


Figure 3.13. Device for heavy metals visual screening. This theoretical scheme displays the potential use of these devices for commercial applications.

Despite other approaches based on cell-free systems[150] are suitable for paper-based devices, the limited number of biochemical reactions available in these systems could limit the creation of complex devices involving multiple circuit components. For instance, it has been demonstrated that very simple but useful biosensors can be easily implemented in paper-based circuits (e.g., mercury detector shown in figure 3.13) and used out of the lab, allowing a rapid estimation of mercury concentration

directly form the visual reporter. Moreover, storing conditions are almost the same as for living organisms and cell-free extracts are far more expensive than standard bacterial cultures in terms of final product cost and scalability.

3.4.6.1 Solution potential versus existing competitors in the market

Lastly, a comparison between the actual solutions available in the market was carried, to determine the potential advantages of the proposed technology. Our solution was named KEYCATCH.

As seen in the table below, heavy metals trace detection is mainly performed using spectroscopic techniques such as atomic absorption spectroscopy and X-ray fluorescence spectrometry, which are the officialised methods for regulatory analysis. These have large detection versatility and high sensitivity but are expensive and require sample preparation and skilled operators to obtain the results. Nevertheless, other options, i.e., electrochemical, optical, and colorimetric strategies have been launched in the market. However, some of them still require sending the samples back to a laboratory or are specialized only in water or in biological samples detection.

Moreover, some of them lack a quantification of the metal being detected and are specific for a group of heavy metals, but not versatile enough to detect all of them. These new detection options in the market range from 20 to 200€ per test and are mainly based on chemical reactions.

	 KEYCATCH	 Commercial kits	 Lab	 In situ kits
PRICE	< 1 €	80 - 180 €	> 30.000	20 - 35 €
TIME				
QUANTIFICATION				
FACILIT				

Table 3.1. List of the main competitors. This table depicts the strengths and weaknesses of the products already in the market and displays the competitive benefits of the devices we are developing in the lab.

As it can be noticed, many options already exist in the market but none of them is fully covering the technical needs or is cheaper enough as threats in our society are still present. For this reason, it is purposed to develop a general architecture for a rapid and *in situ* detection of molecules of interest at a very low cost (< 1€), to not have any price limitation to certain markets or public. Furthermore, the device being presented performs the detection through biologically engineered sensors, meaning the introduction of an innovative way of sensing molecules that is easy to use, not requiring trained personnel. Up until now, some of these biological sensors have been constructed but its poor sensitivity has been a constraint for its further development.

Here, previous experiments revealed a great advance overcoming this limitation, although all sensors should be constructed and tested, to check its sensitivity range. It should finally be highlighted that this way of sensing all type of molecules by living organisms has an enormous scalability in industrial terms, as the prototype would only include a paper strip with the sensor architecture and a small container to keep it sterile. Additionally, this general design offers the possibility of recycling the container for the detection and quantification of any other molecule of interest.

3.5. Discussion

At the beginning of this chapter, we have enumerated several constraints and limitations of current electro-chemical sensors, as well as limitations in living biosensor systems, which offer great advantages compared to standard methodologies but are not reaching the market efficiently. Cell based biosensors offer an extensive sensing capability, being able to detect many different types of molecules of interest being are a great option as a biorecognition element as these are cheaper to purify and make the fabrication relatively simple and cost-effective. However, while electro-chemical systems have evolved towards portable and miniaturized devices with multiple and precise readout options, cell-based system still lack this design flexibility and are difficult to couple with an easy readout method that does not imply complex machineries or infrastructures.

Additional to these intrinsic constraints, the effort towards devising an internationally accepted set of standardized protocols for utilizing and validating a particular cell type or population for a particular application should also be pointed. Thus, more efforts are still required towards unlocking the vast potential of cell-based biosensors in terms of regulatory roadmap before reaching commercialization status.

For all these reasons, the main aim of this chapter was to find an inexpensive, easy, reproducible, and point-of-care system to create biosensors for multiple purposes and capable to detect a broad spectrum of molecules of interest. Thus, having a general and adaptable architecture to bring this high potential biorecognition elements to the market for different applications. As a first approach, two different target molecules have been studied, being heavy metal ions and small quorum sensing peptides, both having well-described receptor systems present in nature. Thus, the intention for these two examples was to display the same architecture design for two extremely different biorecognition elements, exposing the great potential of the design.

The reason for this type of device architecture was the easy adaptability and flexibility based on the molecule to be detected and the ease of use, being able to perform complex analysis without the use of specialized technicians and having an easy on-site readout. It is true that some examples using systems based on colour changes to

quantify the amount of a molecule of interest have been described in literature[79]. However, distinguishing colour changes for quantification can be sometimes tricky so therefore, we came with this quantification based on diffusion patterns, obtaining spots turned ON/OFF depending on the initial sample concentration. Moreover, these results can be seen by necked eye or down the light of a portable transilluminator, being optimal for point of care testing. This offers the possibility to test or perform a first screening without the need of laboratory infrastructures or the use complex techniques.

It is true that additionally to the systems already described, there are some other methods described in literature that use a similar approach but using cell-free extracts rather than whole-cell systems. These systems have a longer durability compared to living cultures. Nevertheless, whole cell devices have demonstrated a good and long preservation when being trapped into paper strips. Moreover, considering the number of reactions being carried, whole-cell biosensors display a greater advantage. Cell-free extracts are expensive to produce and the amount in each reaction is little. Additionally, cell-free extracts were prepared in our lab to assess ON and OFF states from our mercury reporter construct and we only obtained 2,3x fold change.

Wrapping up benefits and limitations of all these systems, in this chapter we have been able to demonstrate a new quantification method based on the combination of different genetic modules and spatial topology. We described two device components, i.e., the transducer and the readout module, each of them involved on the detection and the output production respectively. Moreover, space plays an important role as quantification is made based on the amount of secreted AHL molecules produced by the transducer module, which is dependent on the initial analyte concentration. Thus, the more AHL molecules being released by the transducer module, the longer will be the distance to be reached. Subsequently, longer diffusion distances would imply a higher number of detection spots from the detection module to be turned on.

For the mercury example, this correlation could clearly be seen, obtaining up to seven detection dots turned on and offering an operative range between 10^{-8} and 10^{-5} M of $HgCl_2$.

Although the presented systems are a good alternative for point-of-care quantification of certain analytes of interest, several restrictions have also been observed depending on the ultimate device purposes.

The first thing to consider is time spent for the analysis to occur. Reactions in liquid culture are faster and in the presented examples, overnight inoculation was needed before having the final response displayed in the prototype. Second, although these devices can be stored in the fridge, preservation is no longer than two weeks, being a constraint for commercialisation purposes. Third and more importantly, it has been realised that these devices are optimal when a single measure is needed, for instance, for screening purposes. However, when monitoring is needed, customers do not want to have to take care about the sampling and measuring and want a continuous or semi-continuous measure to keep a good track of what is going on in the studies sample. Thus, although the potential benefits have been described, many challenges still need to be overcome.

Achievements:

- *INNOValora funding from the UPF*

- ***CRAASH Barcelona** a program from Biocat, in collaboration with CIMIT (Boston) and EIT. a 3-month program that helps Catalan-based research teams and startups launch successful device, diagnostic and digital health innovations to improve health and patient care.*

- ***De la Ciència al Mercat**, a course in entrepreneurship from the Catalan universities*

CHAPTER 3

The Art of Living Biosensors – Section 2

OBJECTIVE 2

*In this **second** section we aim to develop a new digital and autonomous solution to track and quantify potential harmful bacteria in situ and create early-warning alerts to prevent further infections.*

[redacted]

3.6 Meeting the gaps regarding pathogen detection

Pathogens are known for playing a key role in disease occurrences. There is a current need in food, medical, environmental, and military markets for rapid and user-friendly methods of detecting such pathogens. Its determination, identification, and quantification is crucial for public health protection[208]. Many approaches have been explored along the years such as enzyme-linked immunosorbent assay (ELISA) [209], electrochemical techniques[210], which are based on changes at electrode interfaces [211,212,213,214], quartz crystal microbalance (QCM) [215], fluorescent spectra [166,216], polymerase chain reaction (PCR) [217], flow cytometry [218], among others, have been reported in literature for bacteria detection.

Although these techniques for bacterial analysis have demonstrated great improvements for being sufficiently sensitive and selective, most still present several shortcomings such as being time-consuming, cost-intensive, or technically complex[219]. Moreover, these techniques also require experimentation and are inflexible in front of different conditions[165,220]. In summary, fast response times and high sensitivity biosensors would address and solve the analytical challenges of the common methods[221].

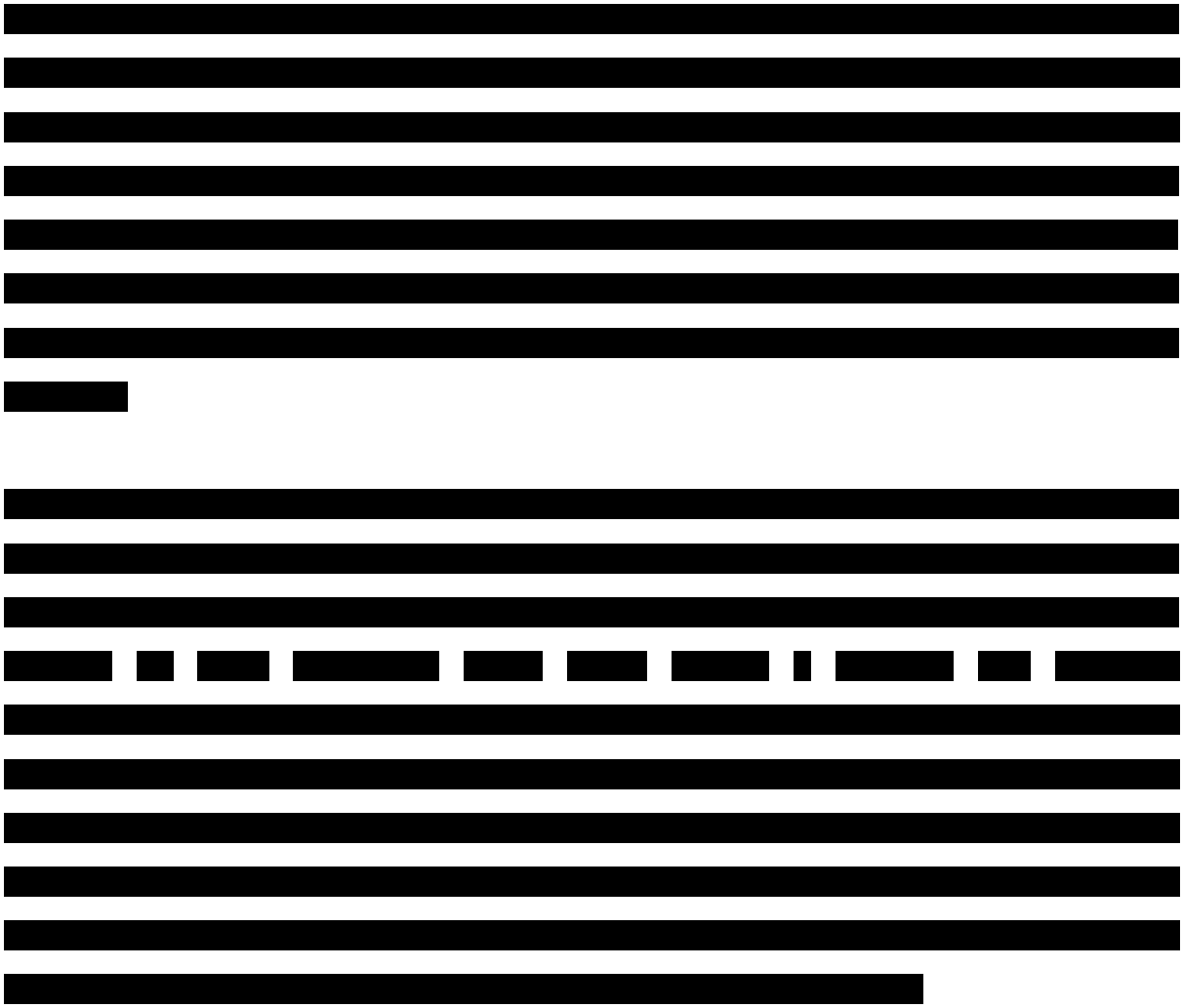
Thus, disposable systems are becoming increasingly important for biotechnological applications[222]. The development of sensors that are small, and fit miniaturized mobile samplers, is an active field of research that requires integration and collaboration between various disciplines[223]. One of the possible approaches to create integrated and autonomous devices are those based on living cells or live microorganisms [224]. Their ability to target specific compound or group of compounds, as well as the quantification and the possibility to determine the expected biological effect due to their exposure is of great interest[225,226]. Moreover, modern bioprocess monitoring demand sensors that provide online and real-time information about the process state.

Bioassays based on live microorganisms, also known as bioreporters, based on bacteria or yeast are, in principle, extremely easy to reproduce and cheaper compared to conventional analytical machines. Because of their small size (1–20 μm) and low population numbers needed for signal detection (10^4–10^6 cells), the integration of

bioreporters into field applicable miniaturized and autonomous devices is a realistic option. Nonetheless, important technical and biological challenges remain before such mobile and autonomous live-biosensor devices can be routinely deployed.

Thus, despite the huge advances made in molecular and microbial detection, there is room for new technologies able to efficiently target industrial needs, without the need of complex infrastructures or specialized personnel. Previously, the chapter started with the detection of chemical compounds, using a single-use device architecture based on signal diffusion for visual quantification.

In this second section, the idea is to move the technology towards the development of pathogen detection systems, developing a whole system, not only a biological setup but also a potential PoC to get closer to the market.



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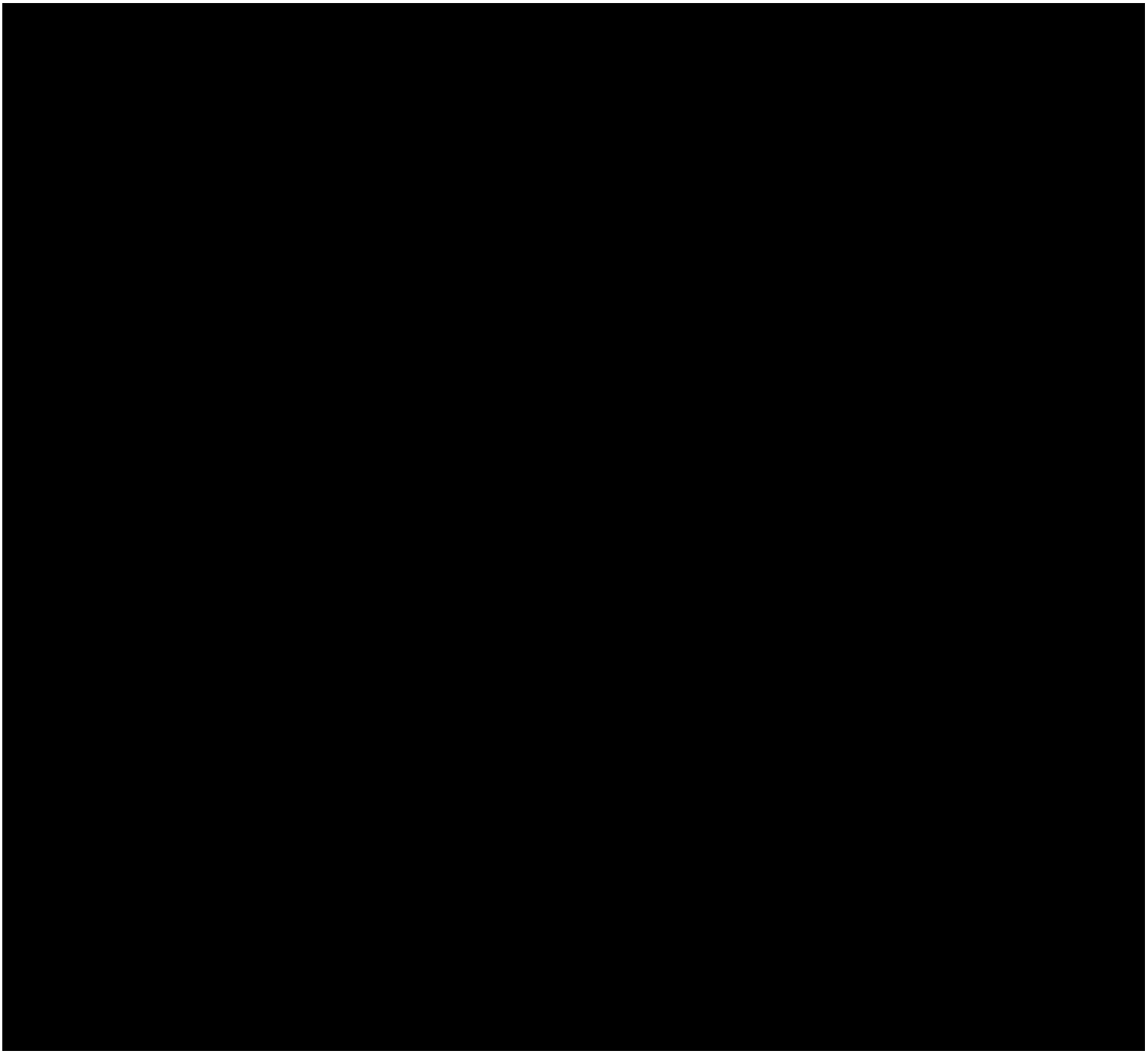

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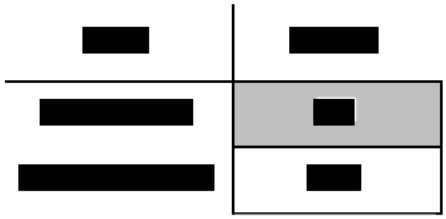

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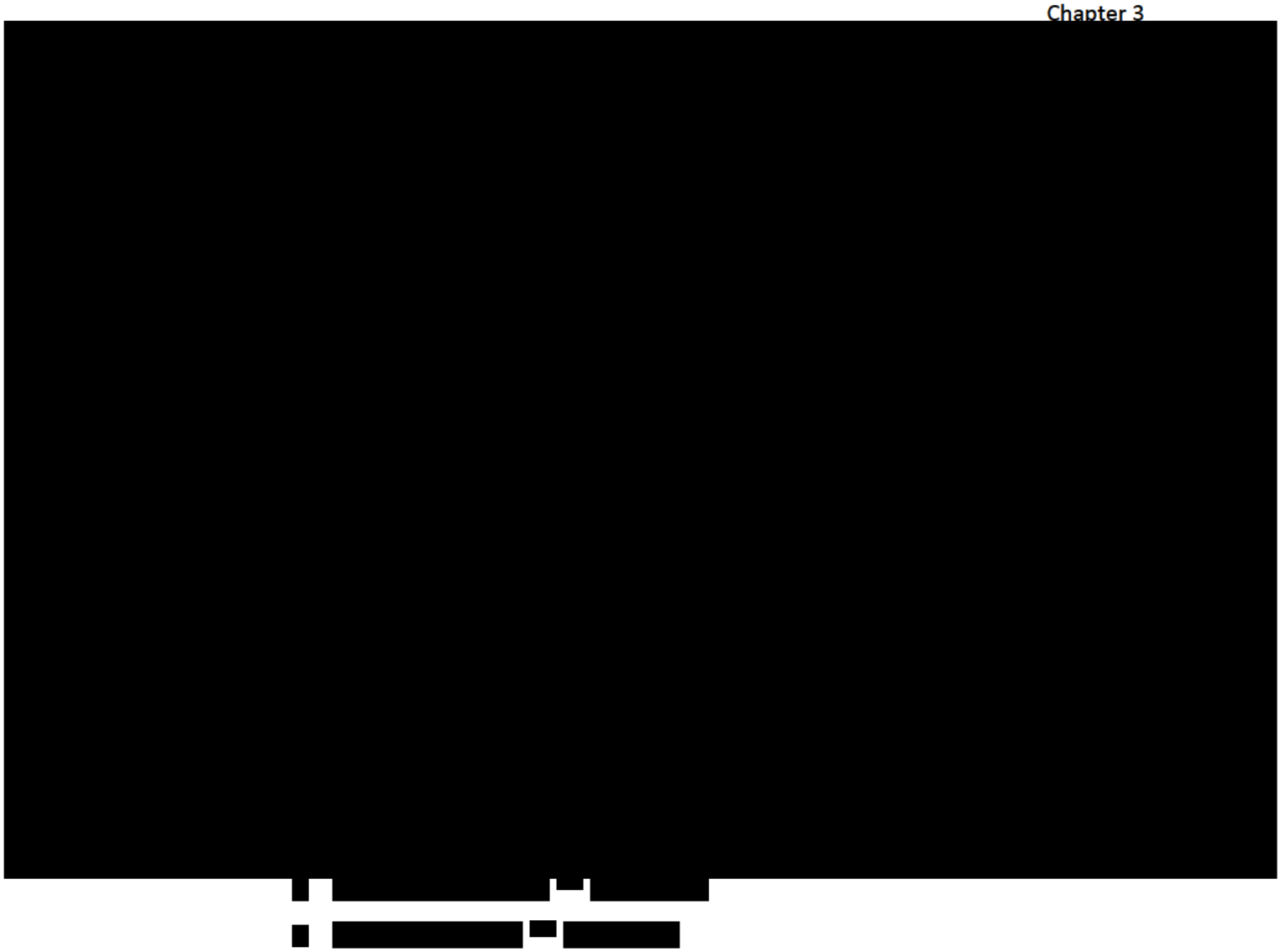

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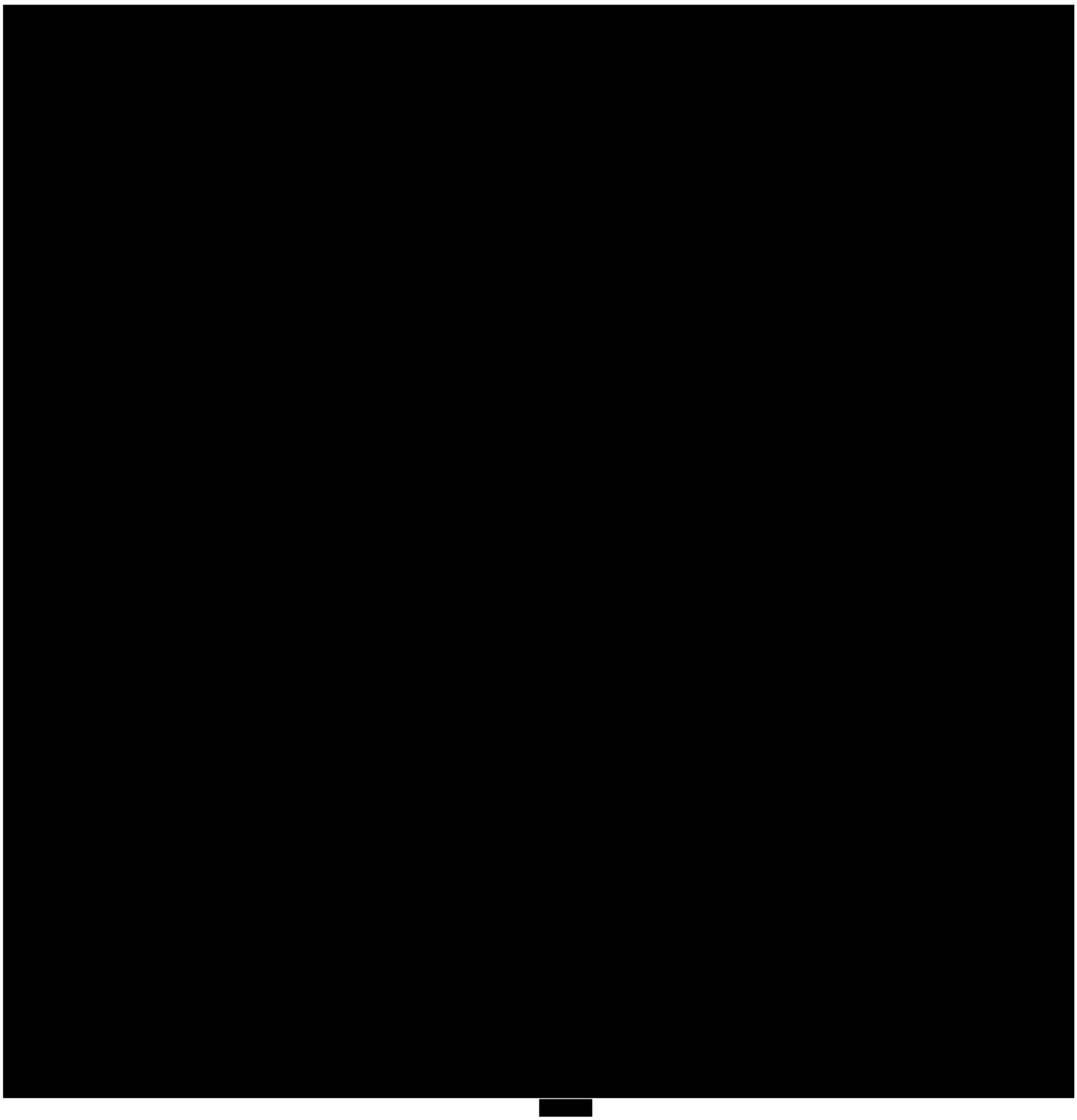

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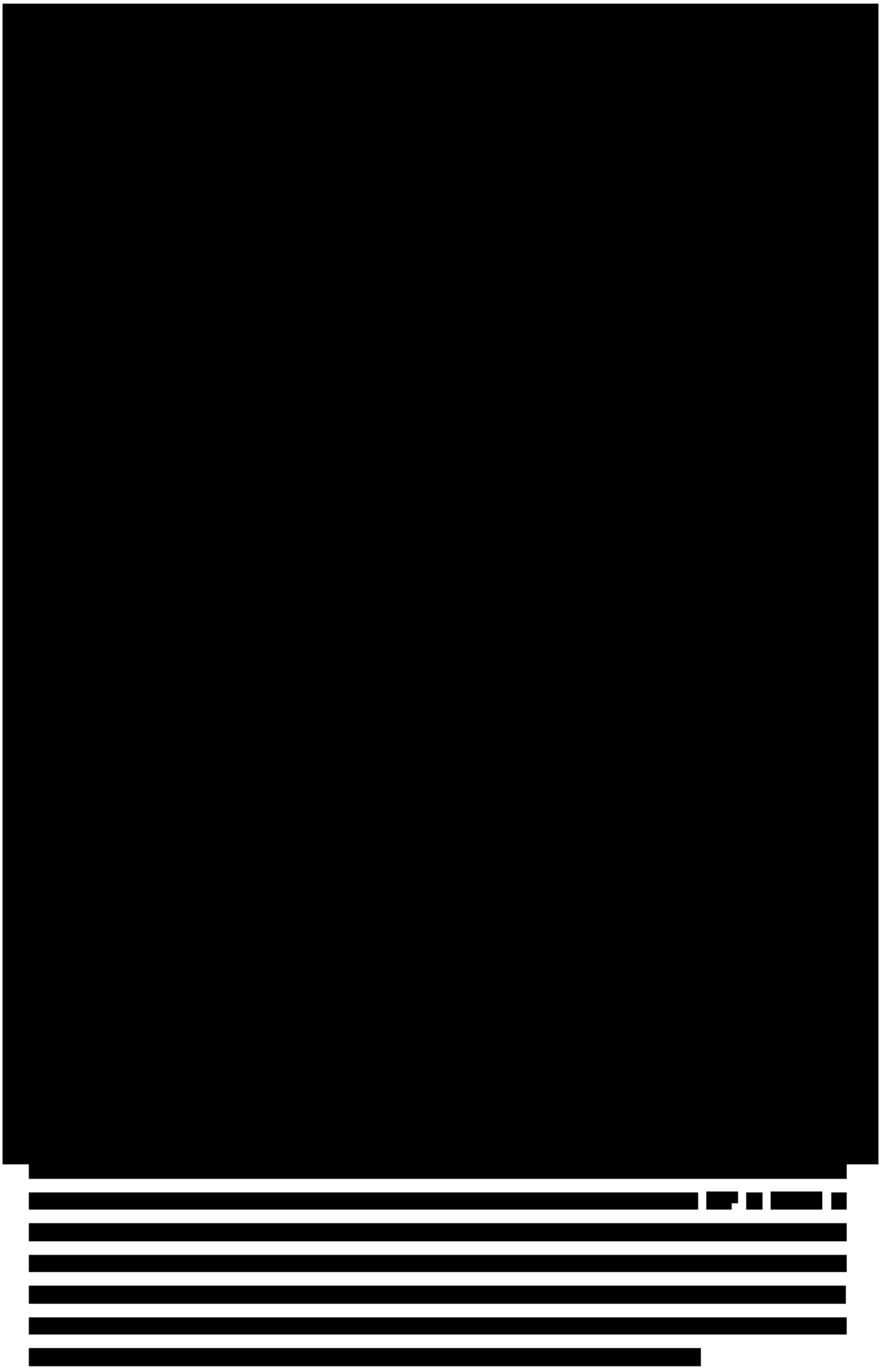

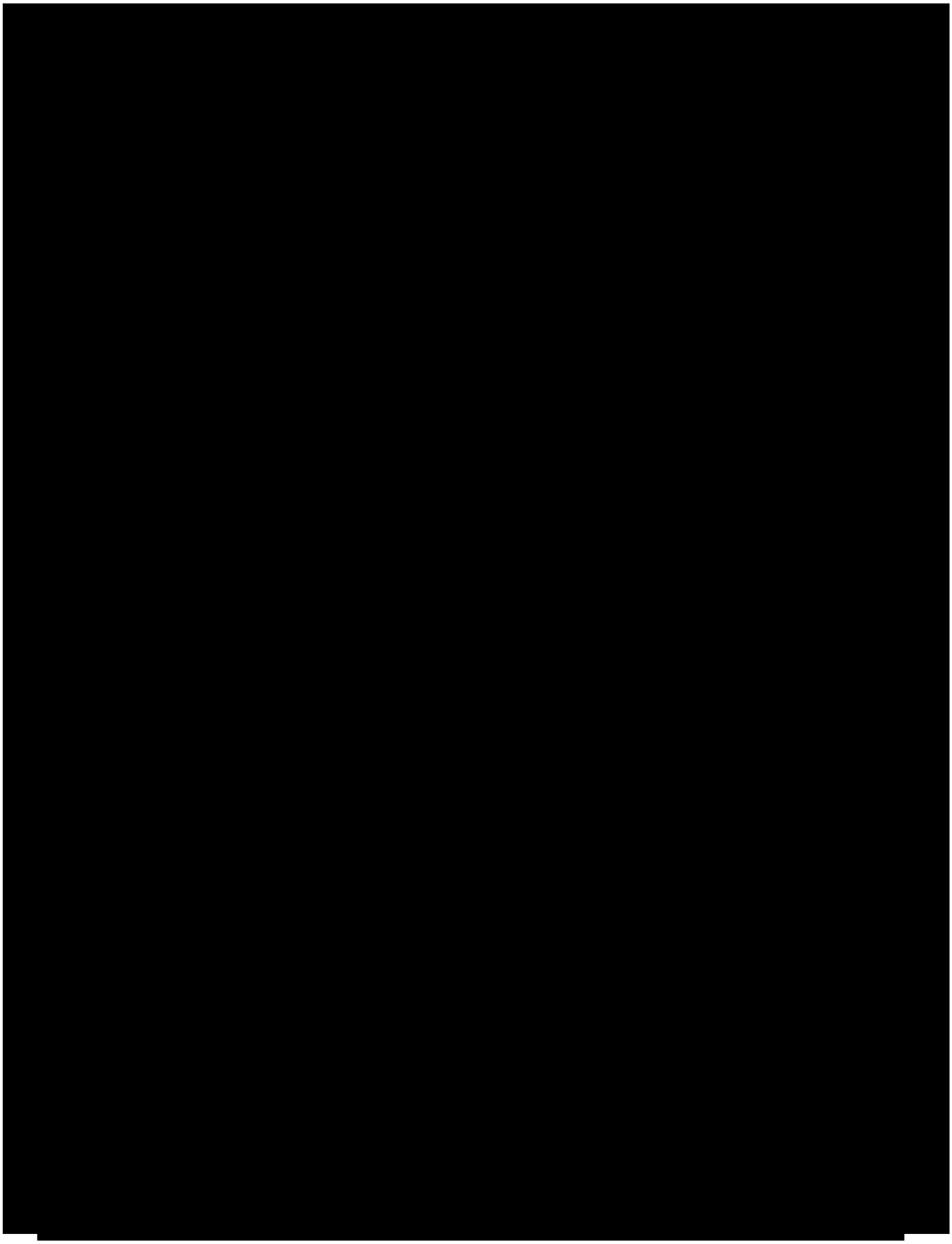

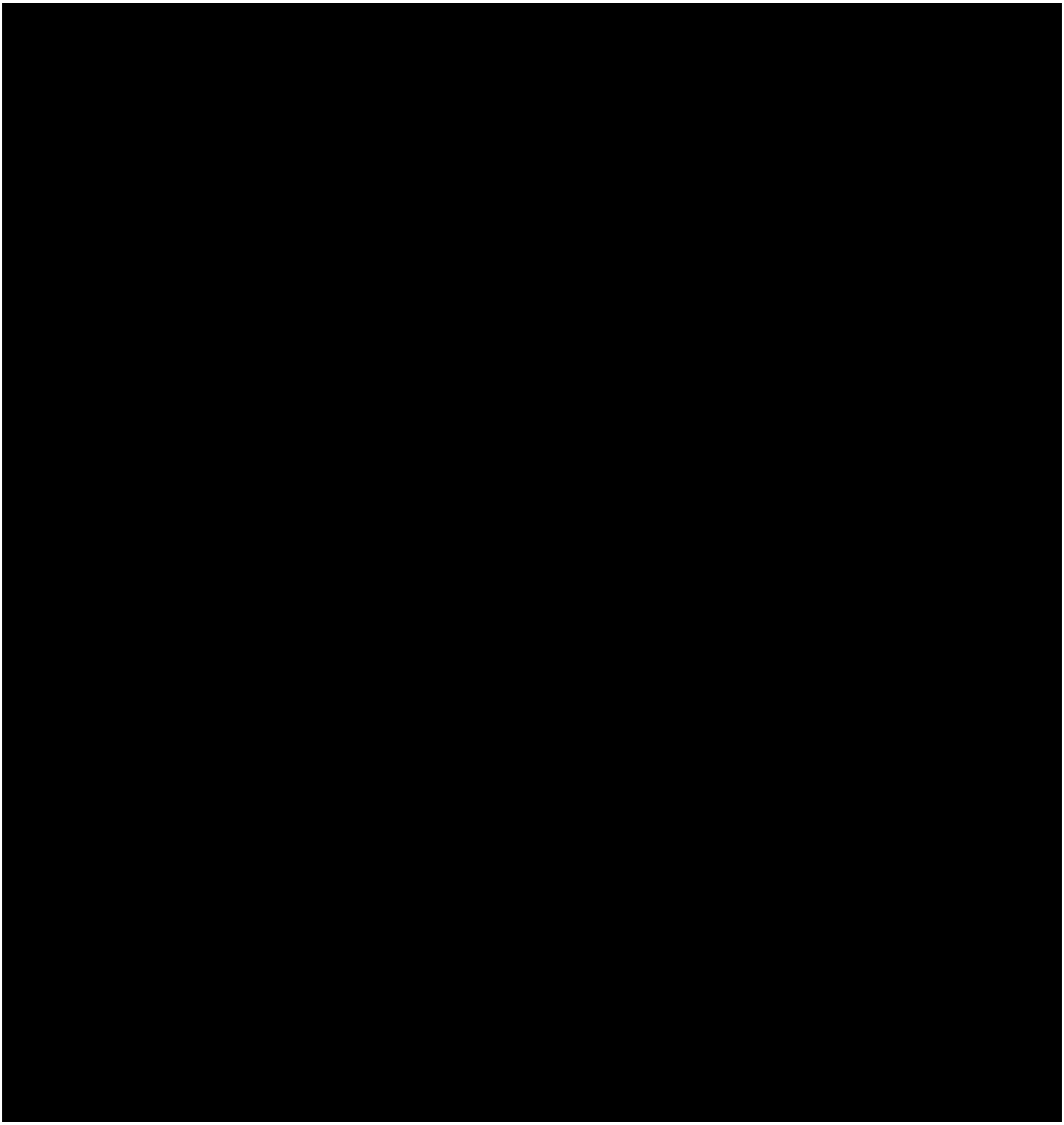

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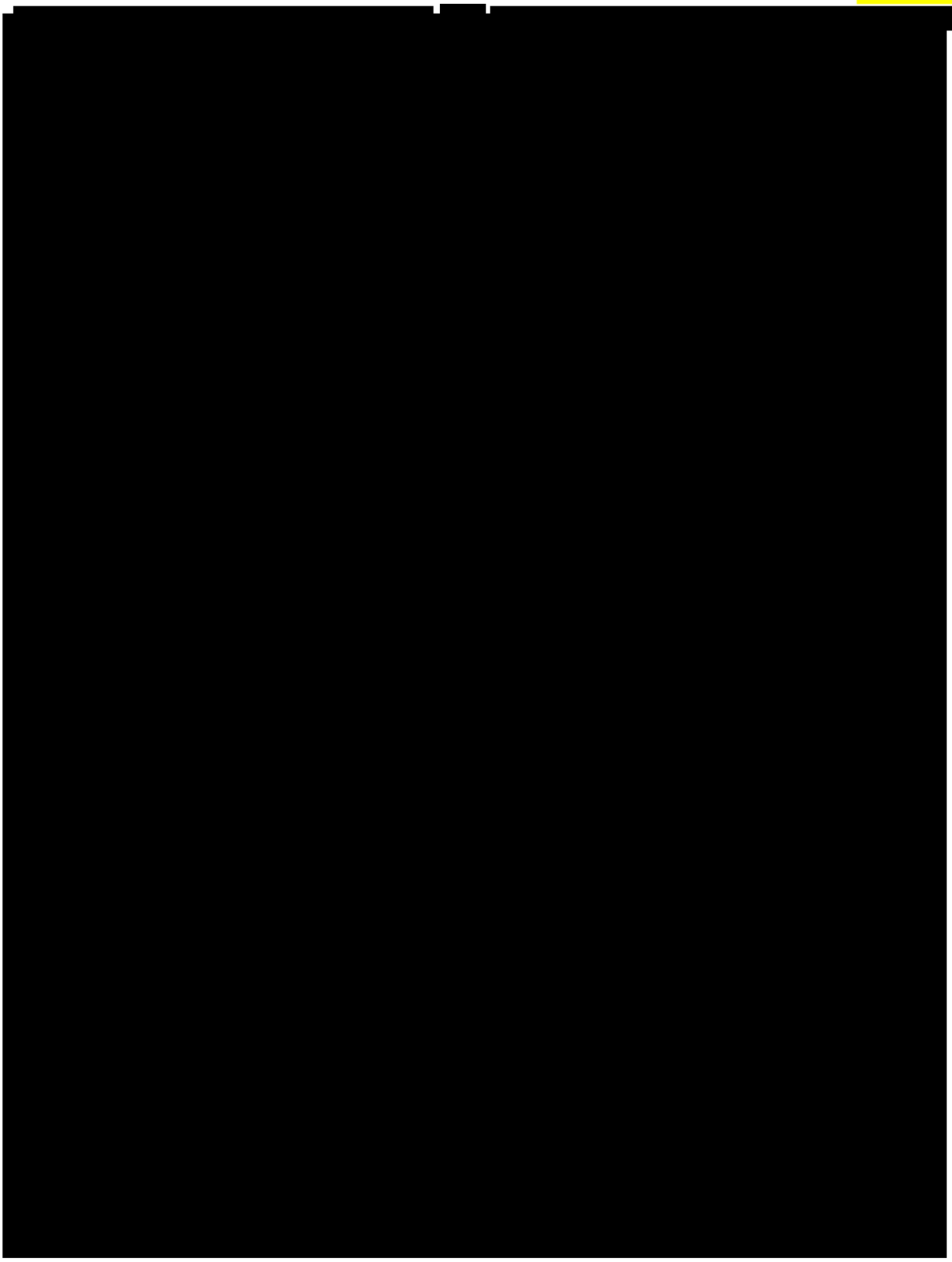

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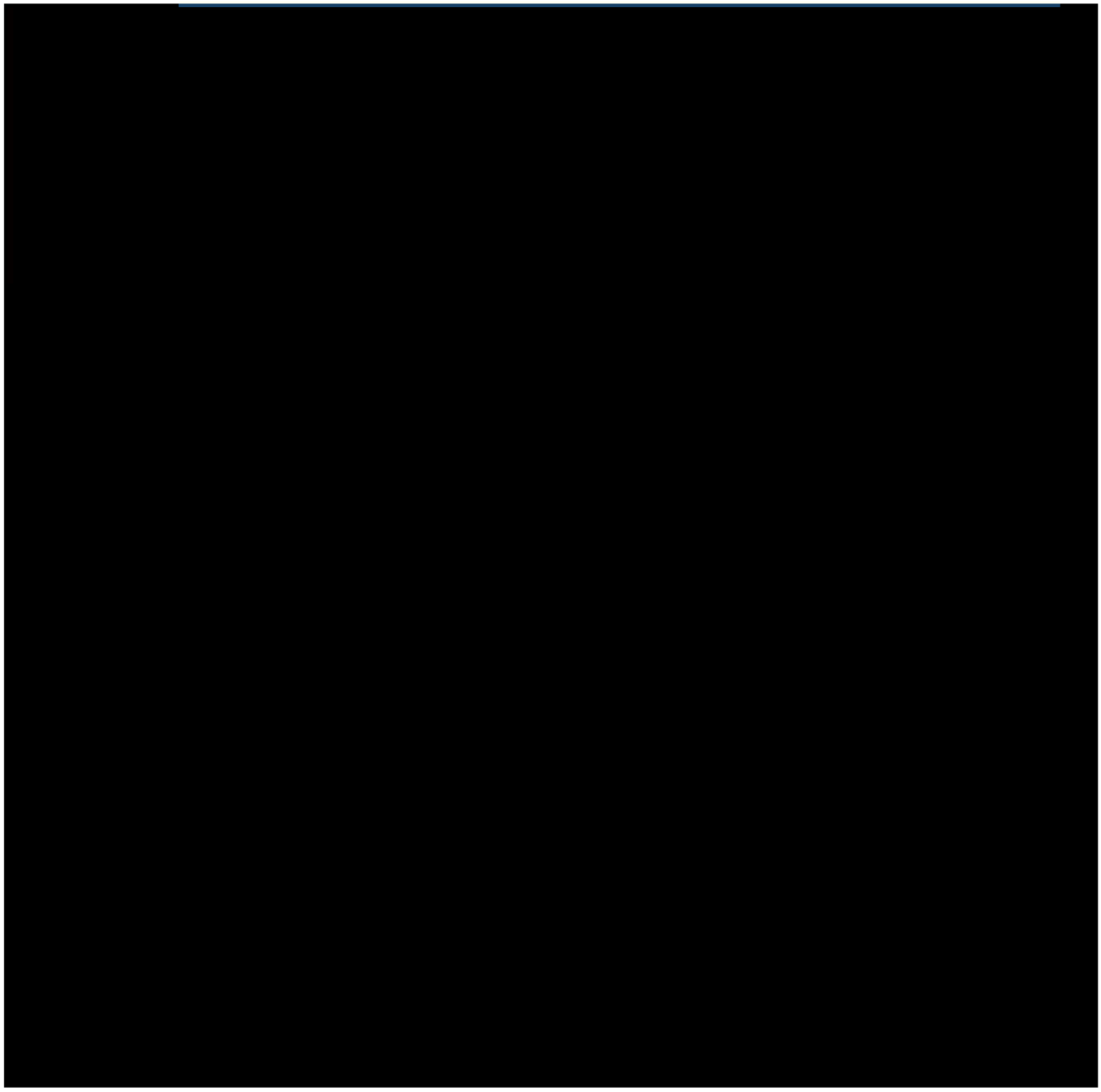

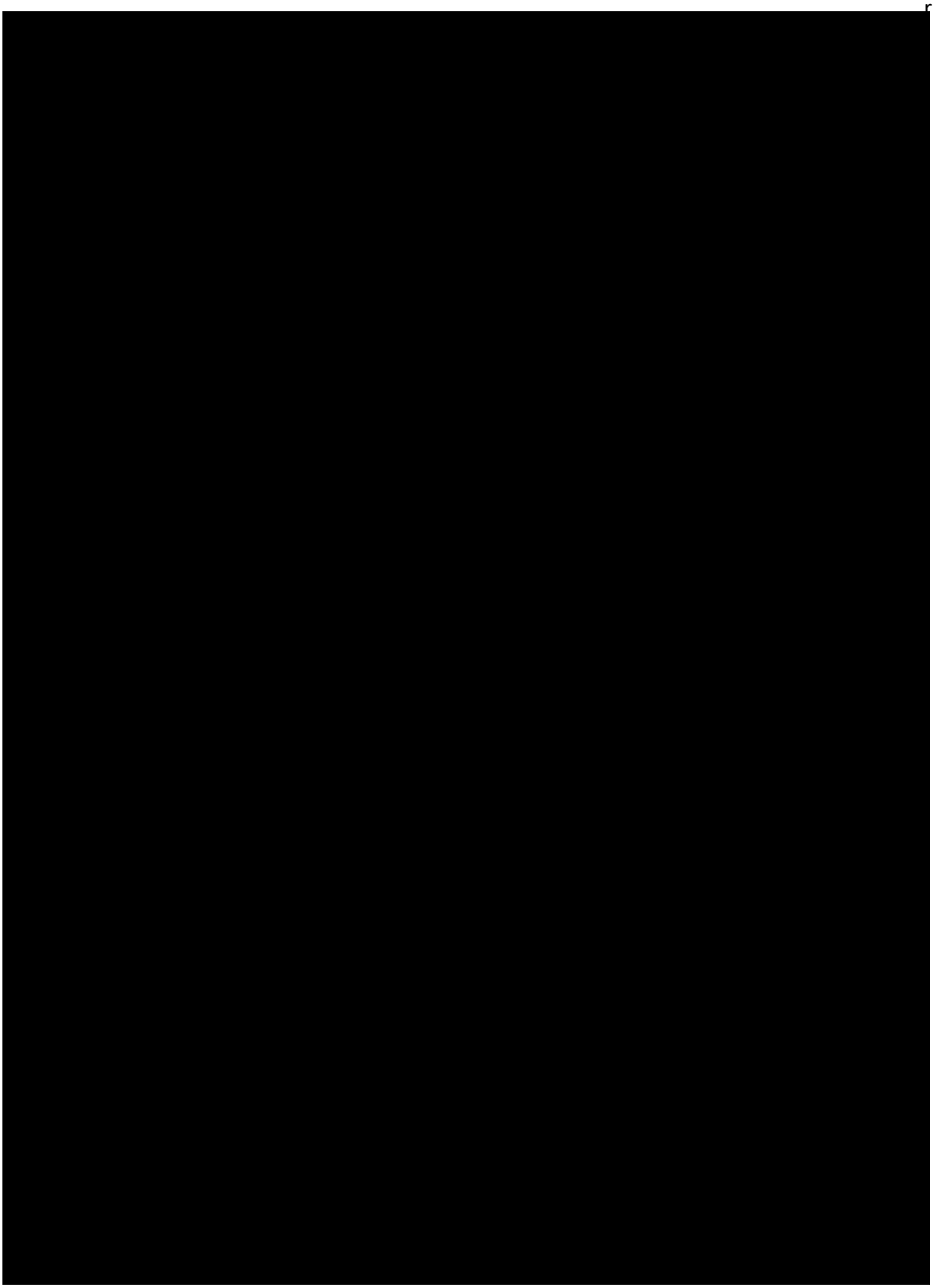

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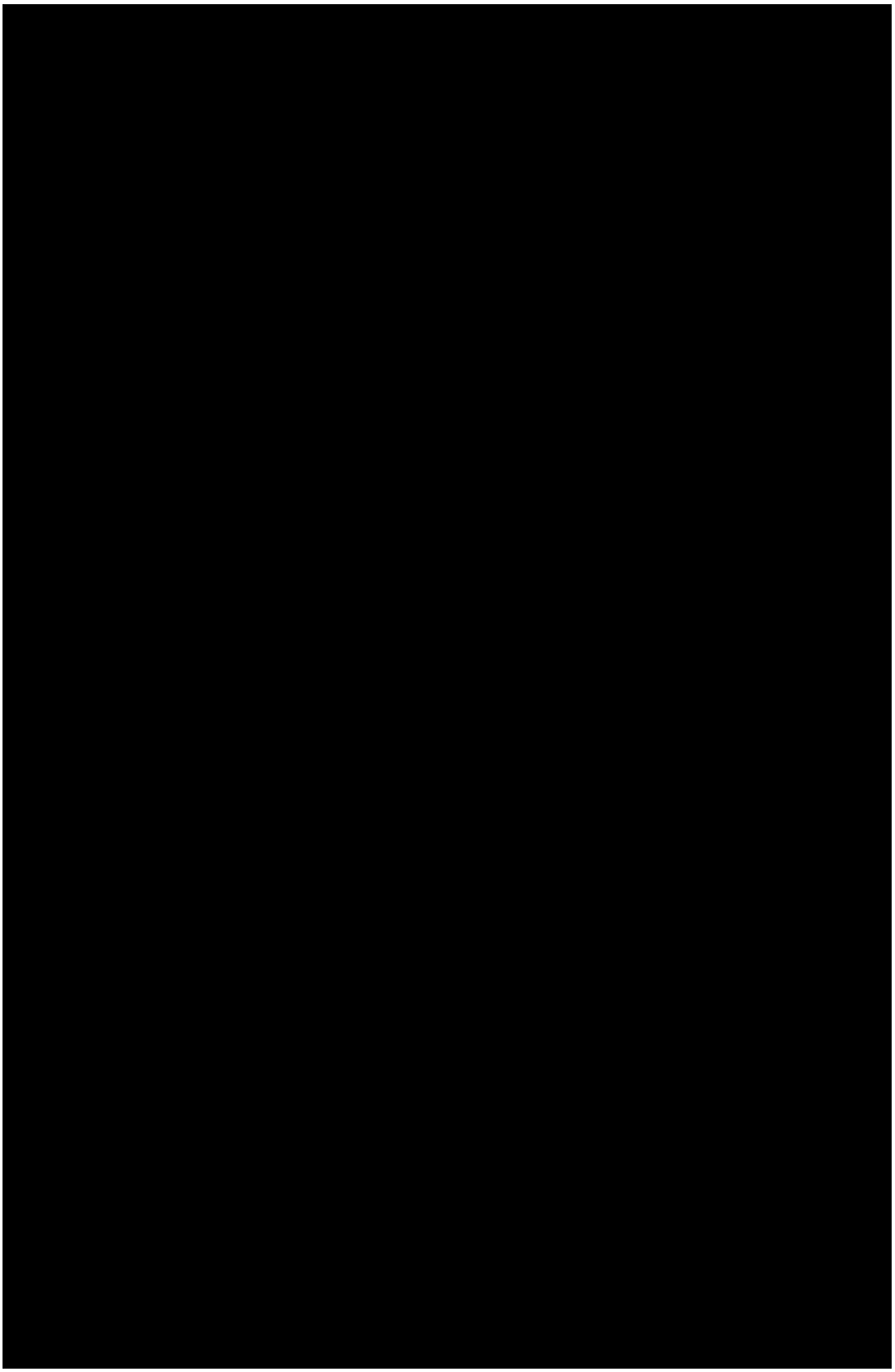

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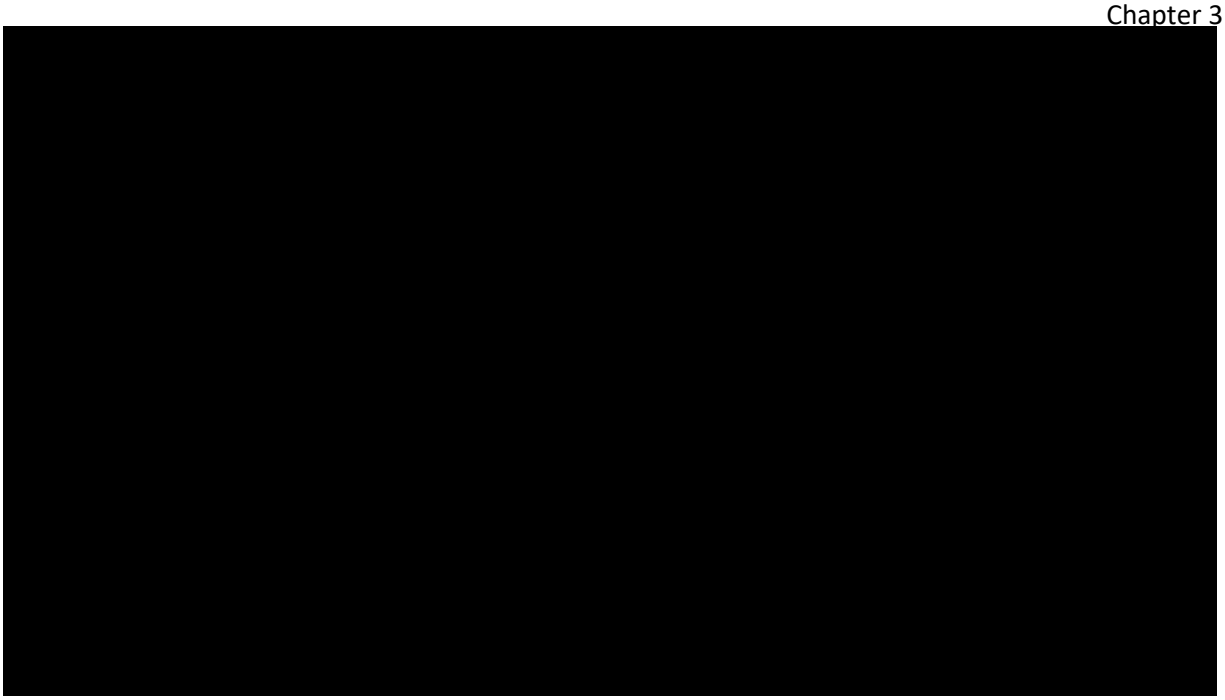

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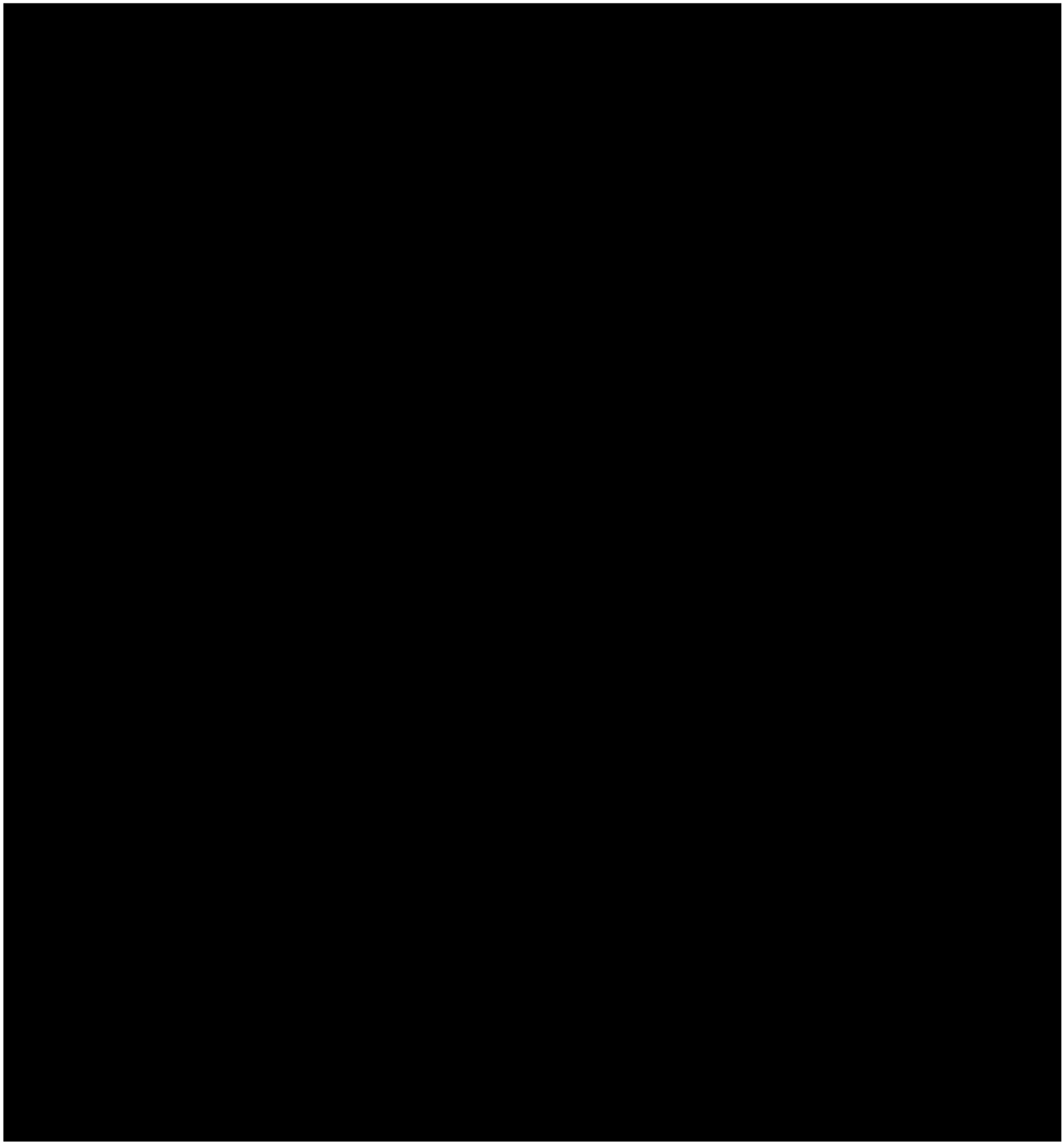

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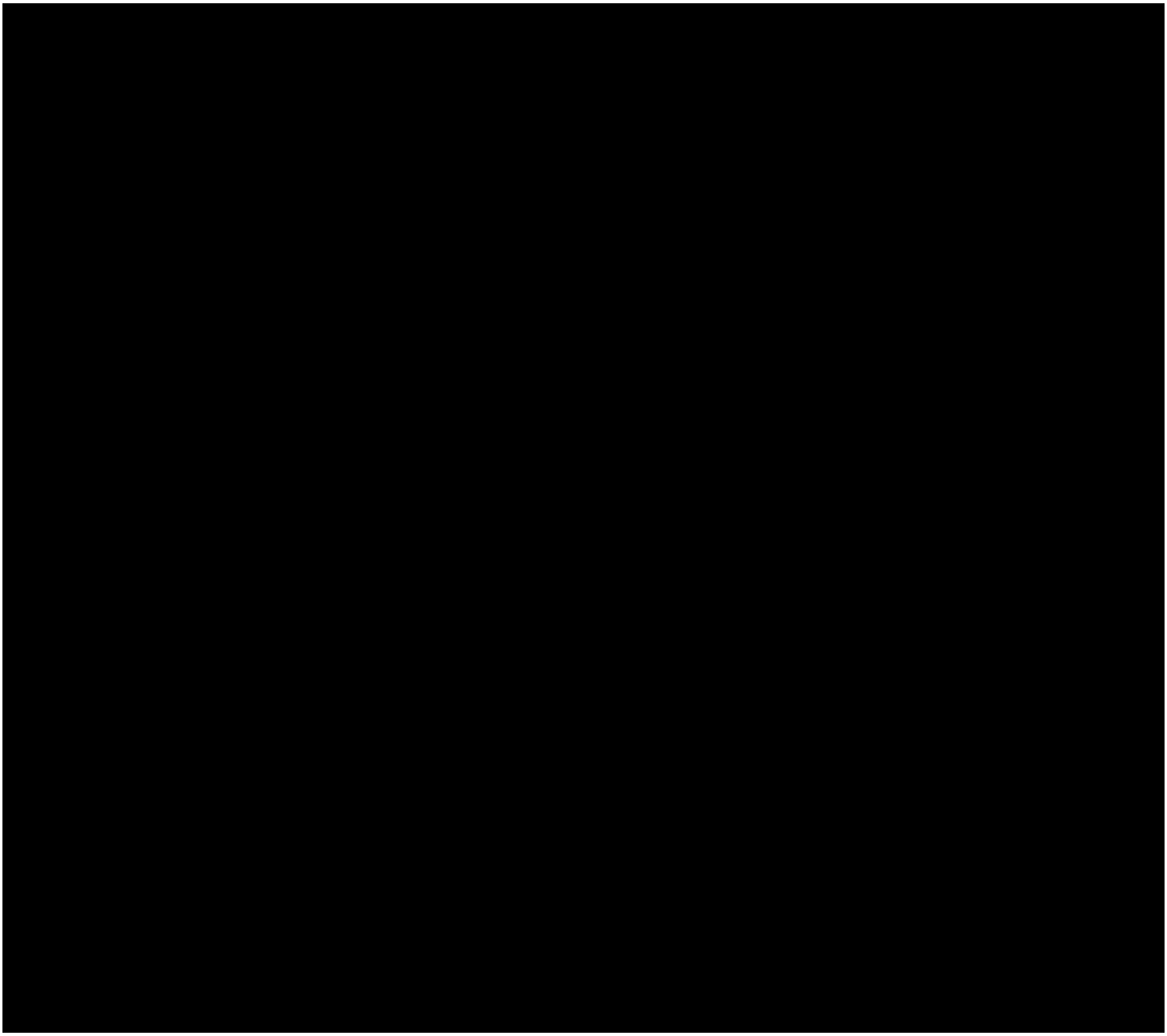

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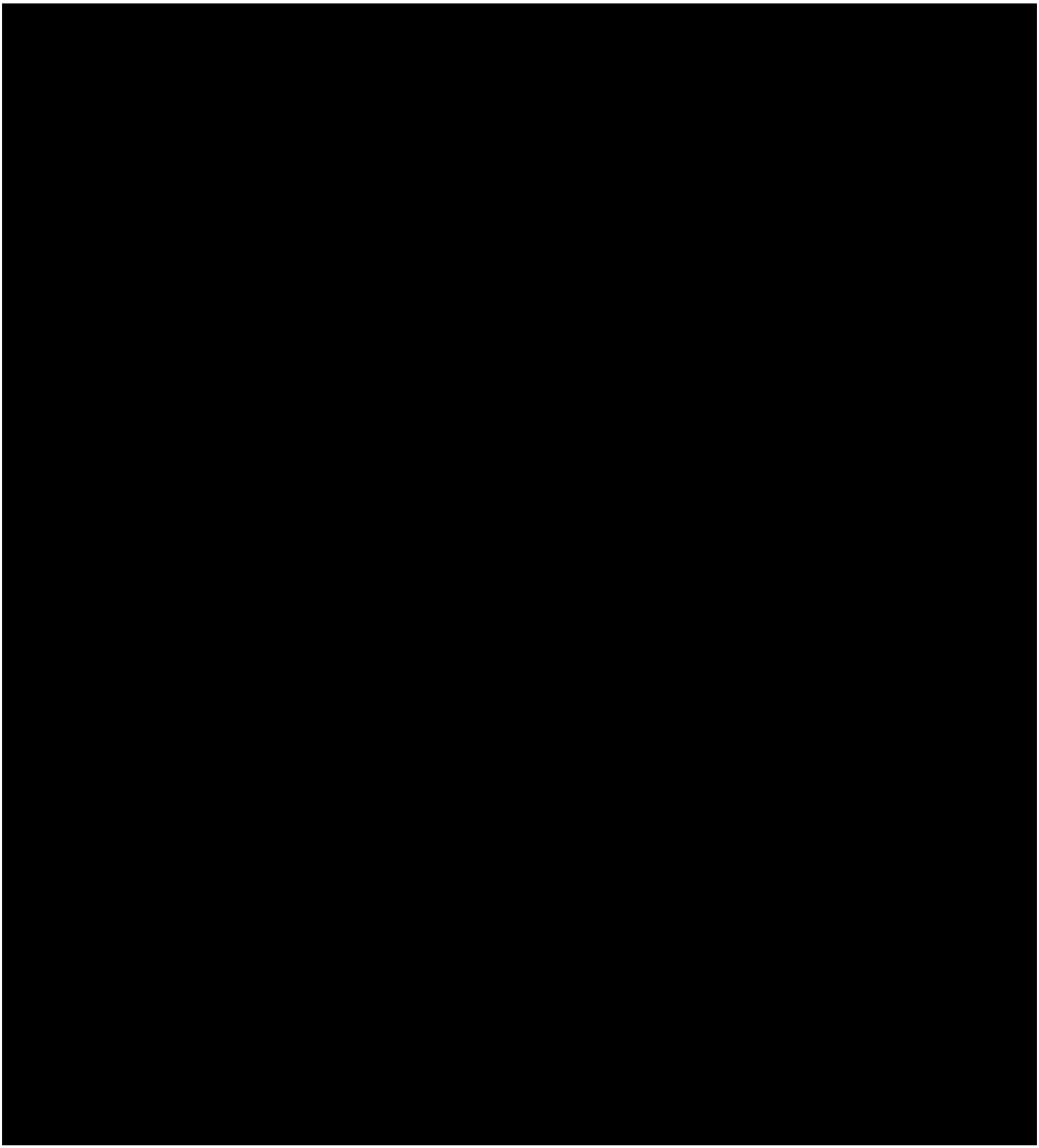

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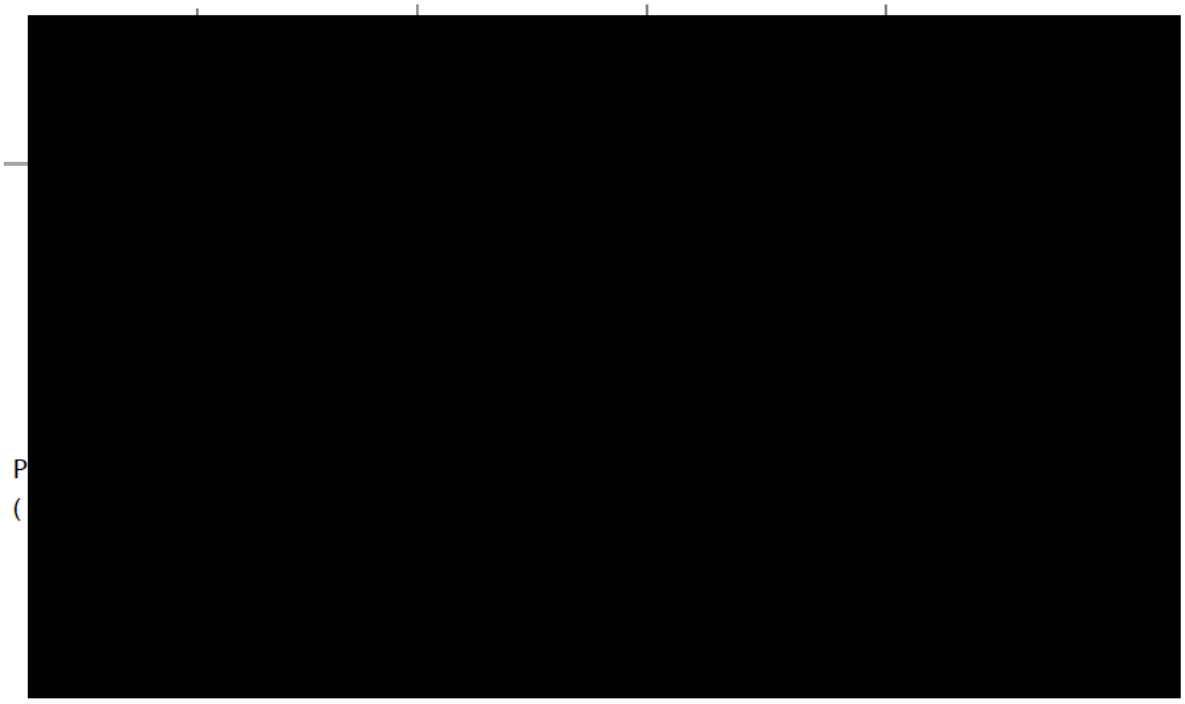

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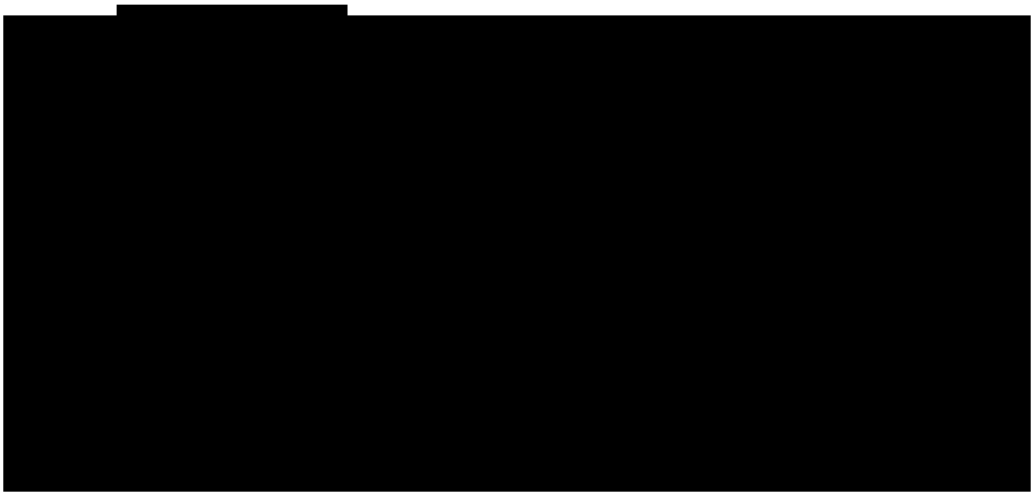

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Achievements:

- *Innovalora funding from the UPF*
- *Selected for **The Collider** (Mobile World Capital)*
- *Selected in **TeamUp EIT Food***
- *Selected in **EIT Jumpstarter***
- *Price for the Best Business Idea from la fundació **Caixa d'Enginyers***
- *Selected in **EmpentaX – ESADE Creapolis***
- *Selected in Hawaii Innovation Studio from **HATCH***

APPENDIX

SESNSA.CHOLERA PROJECT

3.16 Complementary project in underdeveloped countries

This final chapter complement is dedicated to the design of a whole solution as an initiative to reduce cholera incidence in underdeveloped countries. We have presented the project in many grant opportunities such as National Geographic and UPF Solidaria. We are also in contact with an NGO from Guinea Bissau which has expressed its willingness to collaborate with us to run a first pilot to test our biosensors in the field, using water from wells and other water sources.

As already mentioned in this chapter, cholera is disease which remains endemic in Low & Middle Income (LMI) countries such as Africa, Southeast Asia, and the Americas, reaching an annual incidence of 4 million cases.

The project aims to build an innovative, affordable, and easily deployable solution for prevention and real-time monitoring of cholera at the point of need, to achieve a better management of the disease and reduce its incidence.

For that purpose, different actions will be taken: i) Validation of a novel and cheap biosensor to detect cholera in water substrates and other environmental sources. ii) Test deployment and data collection in a cloud-based interface. iii) Generation of insights based on the stored data iv) Training local communities and take prophylaxis measures to improve cholera management and control.

Thanks to our innovative technology based on synthetic biology & engineered cells, these devices will be optimal for rapid *in situ* detection without the need of lab facilities or specialized personnel. Therefore, it will serve as an early detection method to prevent people to get infected due to polluted food or water, reducing the extremely high incidence of cholera worldwide and solving a global public health threat. In fact, the WHO has a roadmap for "Ending cholera 2030" being the early detection in its first axis.

Additionally, in connection with this early detection, we seek to raise awareness and help people in some of the most affected areas to adopt safety habits to also minimize its occurrence. It will be achieved through short and interactive educational workshops

addressed to teachers, community healthcare workers, together with local community engagement to have direct face-to-face feedback. This will allow us to have a positive impact on society making use of our technology.

1. **Validation of our novel biosensors in a relevant environment. Implementation of our biosensor in different locations of our fieldwork to characterize its features.**

Two different engineered bacterial strains have been created in the lab. One of them is capable to produce the non-toxic CAI-1 diffusible autoinducer molecule, mimicking a non-pathogenic version of the Vibrio cholerae strain. On the other hand, we have other engineered bacteria carrying the detection system capable to detect this molecule and produce a visible and quantitative output. After obtaining positive results in a controlled environment, we sick to validate our biosensor prototype in a relevant fieldwork to test its functionality, i.e., sensitivity, specificity, etc. To do so, we plan to implement our biosensor in different location points (water ponds, wells) to collect samples to analyse.

2. **Digital platform for data integration. Automatic system to allow the correct data processing and interpretation.**

An interface to send the information given by the biosensor must be created to then be able to store and manage all the collected data. All this information will be optimal to create heatmaps with relevant “hotspots” where some actions would need to be taken to avoid possible cholera outbreaks. Moreover, the digitalization allows to make predictions, determine the concentration of bacteria in the analysed sample and make the information run to reach the maximum people possible.

3. **Prophylaxis actions. As a prevention method, different strategies will be taken to avoid people to get sick.**

Different prophylaxis actions will be taken in areas showing presence of vibrio cholerae. First, bleach dosages will be poured in polluted water sources. Moreover, antibiotics will be distributed to the population as a preventive method.

4. **Educational actions. In parallel to the prevention strategy, educational workshops will be organized to have a better impact on cholera management.**

4.1. Local research on cholera situation.

Do some local research on cholera situation, receive feedback from local communities, check water resources, availability, and sanitation, together with local experience of sick people and their families. This way, it will be easier to detect the real needs.

4.2. Community face-to-face feedback.

Community face-to-face feedback about our technology and prototype analysis by local people to see the potential but also to detect possible weaknesses to adapt our solution to the current needs.

4.3. Educational project in different schools for teachers to further disseminate the given knowledge in some of the most affected areas reporting cholera outbreaks.

Educational project in different schools for teachers to further disseminate the given knowledge in some of the most affected areas reporting cholera outbreaks. Therefore, people from these places will be able to manage areas with polluted water and to promote protection and conservation of the ecosystem to avoid their situation to be worsened.

However, when considering this initiative, we realized that the storage of these biosensors was not optimal for underdeveloped countries, and we decided to look for a slightly different approach based on our engineered bacteria. For it, we designed a detection tube filled with our detection construct in a lyophilised state, i.e., powder. The storage is much easier, and it can last more time. As we know that in many places the electricity remains a challenge, this lyophilised powder will be refrigerated with a small fridge taking the power from solar panels.

This detection tube will be filled every time with the water to be analysed and we will create a small box that will be optimal to cultivate the sample for a fixed time. This chamber will have the appropriate temperature, shake, and light conditions to be able to perform the detection at the same conditions every time.

The last element being modified is the reporter used. We decided to switch GFP to AmilCp[274], a protein that comes from reef and produces a strong blue colour. Then, the detection will be performed visually, having a colour palette that will correlate different blue intensities with different V cholerae concentrations.

After the detection, the water will be washed with bleach to a container and reused for the next measure. The pipeline to undertake the detection is depicted as following figure (Fig.3.51).

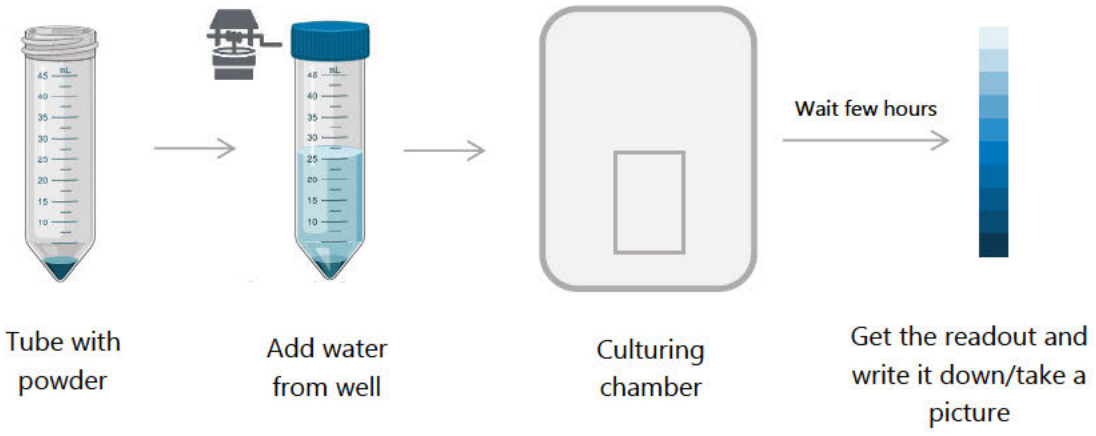

Figure 3.51. Early detection of V cholerae in underdeveloped countries. The idea is to have tube with powder from our lyophilised cells. Then, a sample from a well or another water source will be taken.

Afterwards, a small culture will be performed during a pre-defined time until checking the blue color intensity to assess the obtained result.

The diagram displayed in figure 3.52 shows the general idea of the whole initiative.

Figure 3.52. Whole initiative for ending cholera, improving prevention in any region.

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CHAPTER 4

Discussion and Conclusions

Since the genomics revolution and the rise of systems biology, researchers have been envisioning the time when we would be able to rationally engineer microorganisms. Then, in the 90s, Synthetic Biology was established as a new discipline, with a strong engineering compound[17].

Synthetic Biology offers innovative approaches for finely tunning new biological systems or re-designing existing ones for multiple purposes. It has been described as a disruptive discipline which can deliver solutions in as diverse fields as global healthcare, agriculture, manufacturing, and environmental. Although there is no doubt of its enormous potential, a general perception exists regarding the lack of already existing tools or devices to cover problems in some of the already mentioned fields[14].

Currently, societies are highly industrialized, have poor environmentally friendly habits and are overusing natural resources or intensively exploiting others, such as agriculture or fishing. This is threatening the stability of our ecosystem and our planet. For this reason, in 2015 the United Nations traced a plan to cover 17 Sustainable Development Goals, with the aim to develop new technologies and solutions to transition to a more sustainable society[275].

Interestingly, when analysing Synthetic Biology, we realize that it can take a major role, as it can cover some of the citated Goals such as: i) reducing the use of harmful chemicals for biologically-based alternatives, ii) bioremediation to remove pollutants in environmental sources, iii) increase productivity in some industries such as crop production or fish farming, as well as improving health, iv) replace synthetic, non-renewable materials for more environmentally-friendly ones[276].

However, although the huge technological advances made for gene synthesis, process automation, and miniaturization, which are rapidly transforming this field, standardization is still missing in many aspects, not only in the design but also on the workflow, the measurement, and the delivery of solutions in this infant field[6].

Furthermore, all the benefits we can take from Synthetic Biology approaches can damage our society if developing countries start producing synthetic versions of natural products and thus, increase global inequality, as well as biodiversity

threatening or reduction with the incorporation of genetically modified organisms in the environment. For this reason, legislation for confined-use synthetic biology solutions is less restrictive as for deployable solutions[277,9].

Thus, moving to the first practical example of this thesis, in **Chapter 2** we have analysed the main constraints of synthetic circuit design to come up with a new methodological approach.

Synthetic Biology rationally designs and engineers biological systems to achieve new functions such as a living Toggle Switch[278] or a Repressilator[132]. With these examples, it was demonstrated that natural genetic components could be re-wired and used for specific desired goals. However, many challenges have appeared since then.

To date, most cellular computational devices have been designed according to standard architecture that organizes their components into three different layers: the input layer, where external signals are detected; the processing layer, where computation is performed; and the output layer, where the final output is produced[279,92,93,94]. However, although many advances have been achieved, such the creation of standard synthetic part libraries or the use of circuit design software inspired on electronic design automation (EDA)[97,98], many constraints such as the so-called wiring problem[119,280,281], the genetic complexity[281,122,282], the metabolic burden[283,284,285,106] or the presence of random fluctuations inherent from biological systems[286] still limit the development of complex devices, which compromise the development of end-user applications[287,288,289 290,291,27].

Seeing these limitations and by taking inspiration from the fundamental basis of transistors, which have revolutionised modern electronic devices and are considered the biggest inventions of the 20th century, and fusing these concepts with printed electronics, a new methodological approach has been developed for the construction of printable cellular devices, i.e., digital or analogue, for different purposes. This approach is appealing because it provides circuits with: i) higher temporal stability, ii) larger reproducibility, iii) easier experimental setup, using different combinatorial strain compositions, and iv) low-cost production.

Printed electronics (PE) has emerged as one of the key technologies for electronics and electrically controlled machines and equipment, as It merges electronics manufacturing and text/graphic printing, allowing thin, wearable, flexible, cost-effective and environmentally friendly final products[292]. Adding organic electronics onto paper surfaces would be of great interest for retail chains and transportation companies which seek a printed electronic technology to provide better safety and security features on packages and automatically track and trace products all the way from the manufacturer to the end customer. In addition, printed electronics could potentially guide the end-user to properly use the product and to guarantee brand authenticity[293,135]

Paper is manufactured in a wide variety of categories (*e.g.*, as printing paper, wrapping paper, writing paper, drawing paper, specialty paper) and is thus available with a wide range of properties and specifications. One of the challenges associated with the use of paper as a substrate for electronic devices is its often significant surface roughness[134].

The implemented approach for this new methodology integrates space as a new computational element where elements are physically separated between them. This avoids any type of undesired interaction and allows them to wire thanks to diffusible molecules, which mimic its counterpart in electronics, i.e., electricity. By predefining spatial patterns using a multi-branch architecture, we have demonstrated that we can build highly complex circuits such as the design of 3-input gates for digital computation and a band-pass filter, for analogic computation, by just using the same pre-defined set of computationally active elements.

Moreover, we have been able to develop a methodology that can automate circuit construction. The only downside of it is that distances need to be manually established. Nevertheless, once having the right distances between elements, these circuits can be replicated as many times as possible, knowing that, additionally, these circuits can be stored in the fridge during almost two weeks and still be functional, or can even be freeze at -80°C for its further use.

From this first work, which fuses a more conceptual definition of general architecture design with practical examples for its validation and further potential exploration, some new concepts will be introduced in future works such as using the same principles of spatial patterning to implement different computations thanks to light patterns[294].

Furthermore, many potential applications have come up which will be explored in the future. For instance, this approach can potentially be interesting for highly complex and multi-parametric disease diagnosis. Thus, we would define the parameters to be analysed and its correlation with a specific disease, i.e., presence/absence, high/low levels, and would create the specific circuit logic needed to have the final output result. This can be interesting in some diseases such as preeclampsia or for some diagnosis such as antibiotic resistance for a certain pathogenic strain. This last application is being developed by ARIA, the UPF team which participates in the 2021 IGEM competition from the MIT. They are creating a paper-based array which contains well-defined biological Antibiotic Resistance hallmarks that can be identified thanks to CRISPR-based biosensors that release an optical signal (fluorescence) when interacting with them.

Other potential approaches explore the creation of bioactive matrices which can be used as smart portable devices for measuring or for delivering purposes[295,296].

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5.1 Main research findings

Chapter 1 – 2D printed multicellular devices performing computation based on spatial arrangement

- Establishment of a multicellular approach which encodes information in the modulation of a diffusible signal in 2D surfaces
- Biological computation, either digital or analogue, thanks to a multi-branch approach architecture. This approach enables to systematically map any arbitrary truth table
- Digital computation through the experimental validation of 2-input and 3-input gates
- Analogue computation with the different modulatory levels obtained from our base-like transistor counterparts
- Stamping templates used to automate circuit creation from a simple and low-cost approach.

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5.2 Spin-off one pager – KOA BIOTECH

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[redacted]

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[redacted]

"Genet ca y Eng neer ng the uture"

SCIENTIFIC PUBLICATIONS

During the two-year period of this PhD thesis in the Synthetic Biology for Biomedical Applications Lab, research achievements have been translated into one scientific publication and one patent about to be filled:

Mogas-Diez S, Gonzalez-Flo E, Macía J, (2021). 2D printed multicellular devices performing digital and analogue computation. Nat Commun 12, 1679. DOI: https://doi.org/10.1038/s41467-021-21967-x

Informe de patentabilidad relativo a biosensores basados en tecnología híbrida que combina dispositivos electrónicos con organismos vivos modificados geneticamente para detector patógenos bacterianos. **REFERENCIA:** E20831WW00

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ANNEX 1

S1. Chapter 2

S1.1 MATERIALS & METHODS

S1.1.1 Strains, media, and growth conditions

Cloning and expression experiments were performed in Escherichia Coli Top10 (Invitrogen, USA) and Escherichia Coli ZN1 (ExpresSys, Germany). Cells were grown in Lysogeny Broth (LB) at 37◦C and selected with appropriate antibiotics (chloramphenicol 35 μg/ml, kanamycin 35 μg/ml Sigma, USA). Bacterial strains were preserved in LB glycerol 20% (v/v) at −80◦C. Cells inoculated from single colonies form streaked glycerol stocks were grown at 37ºC with shaking (450 revolutions per minute (rpm)) for about 5 hours until reaching an OD between 0,15-0,2.

Experiments were performed in petri dishes (ddbiolab) pouring 20 ml of LB broth with agar (Sigma Aldrich, USA) supplemented with the antibiotic and the appropriate inducers before depositing the paper strips to print the circuit.

The inducers used were L-(+)-arabinose 98% (Sigma Aldrich, USA), N-3-oxo-tetradecanoyl-L-Homoserine lactone (Cayman Chemical, USA) and Anhydrotetracycline 98% (Cayman Chemical,USA).

S1.1.2 Plasmid assembly

The assembly of genetic construct was carried out using the Biobrick assembly method for molecular cloning and genetic sequences were obtained from Parts Registry (http://parts.igem.org). All constructs analysed in this thesis were built by combining these parts using 3A assembly using the standard four restriction enzymes, i.e., EcoRI, PstI, XbaI and SpeI. All constructs were included in the Biobricks high copy number plasmid (pSB1AK3 or pSB1AC3) and were transformed using a heat shock method.

S1.1.3. Paper properties

The paper used for all experiments was 75gsm White Paper (Pack of 2500) 59908. It has an optimal quality due to its eucalipto globulus fibers mixed with calcium carbonate. This combination allows to produce a 75 g/m^2 paper with necessary rigidity and opacity.

S1.1.4 GFP quantification

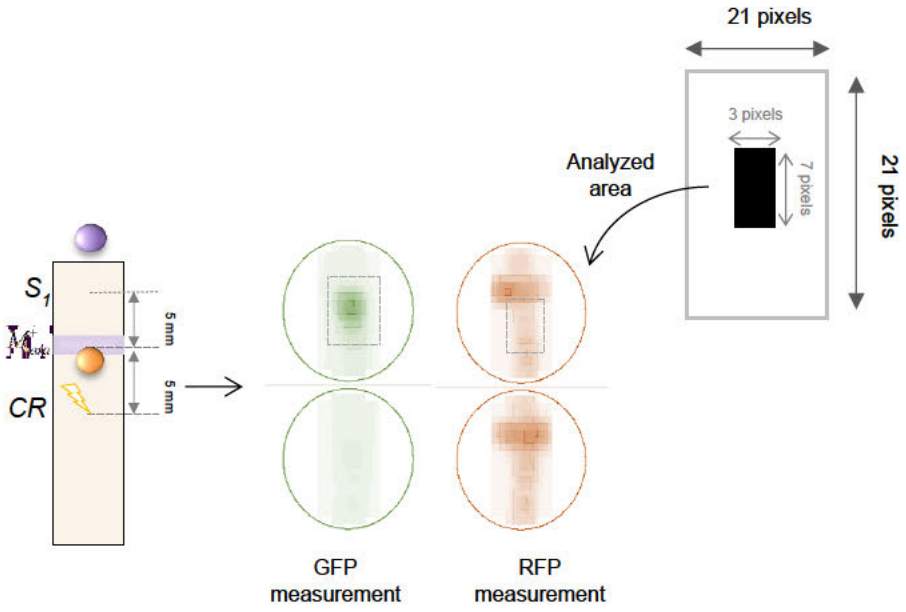
In all experiments, circuits printed in paper were grown at 37°C for an overnight and after 24h, a surface scanning was carried, measuring GFP and RFP intensities. Measures were performed with *Synergy HXT- Hybrid Multi-Mode Reader* (BioTek Instruments, USA).

Fluorescent measures for GFP excitation wavelength. 475∓9 nm, emission wavelength: 509∓9nm. Fluorescence measures for RFP: excitation wavelength: 578∓9 nm, emission wavelength: 616∓9 nm.

S1.1.5 Surface scan analysis of paper strips

After inoculating paper strips, a protocol for scanning paper strips with the already mentioned wavelengths to measure GFP and RFP was created. A schematic diagram is represented in the following image to explain how quantification was carried.

Scans had an area conforming 21x21 pixels. From that area, a smaller one was taken to analyse both measures coming from the GFP intensity and the RFP constitutively being produced. Figure S1.1 displays these processes.



Supplementary Figure S1.1. Response quantification through paper strip scanning. Once having the system response, strips were scanned, using the specific wavelengths for GFP and RFP measures. From all the scanned area, a smaller rectangle, i.e., 3x7 pixels, was taken to normalize GFP measures with the amount of RFP produced.

S2. Chapter 3

S2.1 MATERIALS & METHODS

S2.1.1 Strains, media, and growth conditions

Cloning and expression experiments were performed in Escherichia Coli Top10 (Invitrogen, USA). Cells were grown in Lysogeny Broth (LB) at 37◦C and selected with appropriate antibiotics (chloramphenicol 35 μg/ml, kanamycin 35 μg/ml Sigma, USA). Bacterial strains were preserved in LB glycerol 20% (v/v) at −80◦C. Single colonies obtained from streaked glycerol stocks were inoculated and the cells were grown overnight at 37 ºC with shaking (200 revolutions per minute (rpm)). Overnight cultures were diluted into fresh LB (1/100 dilution) and grown for 5 hours. Diluted cultures were loaded into a 96-well microplate (Nunc, Thermo Fisher Scientific, USA) and induced (see below) in a final volume of 200 μl.

The inducers used were L-(+)-arabinose 98% (Sigma Aldrich, USA), N-3-oxo-tetradecanoyl-L-Homoserine lactone (Cayman Chemical, USA), N-butyryl-L-Homoserine lactone (Lab clinics, Spain), N-(3-Oxooctanoyl)-L-homoserine lactone (Merck Life, Spain), N-(3-oxododecanoyl)-L-Homoserine lactone (Merck Life, Spain), Mercury chloride 99% (Sigma Aldrich, USA).

For filtering supernatants from our pathogen-like constructs, we used 20ml single use plastic syringe (Servicios hospitalarios, Spain) and MF-Millipore Membrane Filter, 0.45 μm pore size (Merck Life, Spain).

In the second section of Chapter 3, two *Vibrio* species were ordered, *Listonella anguillarum* ATCC 43306 and *Vibrio harveyi* ATCC 33868. For it, we bought a specific growth media, Marine Broth (Sigma Aldrich, USA).

S2.1.1.1 Concentrated LB

[redacted]

S2.1.2 Lyophilisation process

[redacted]

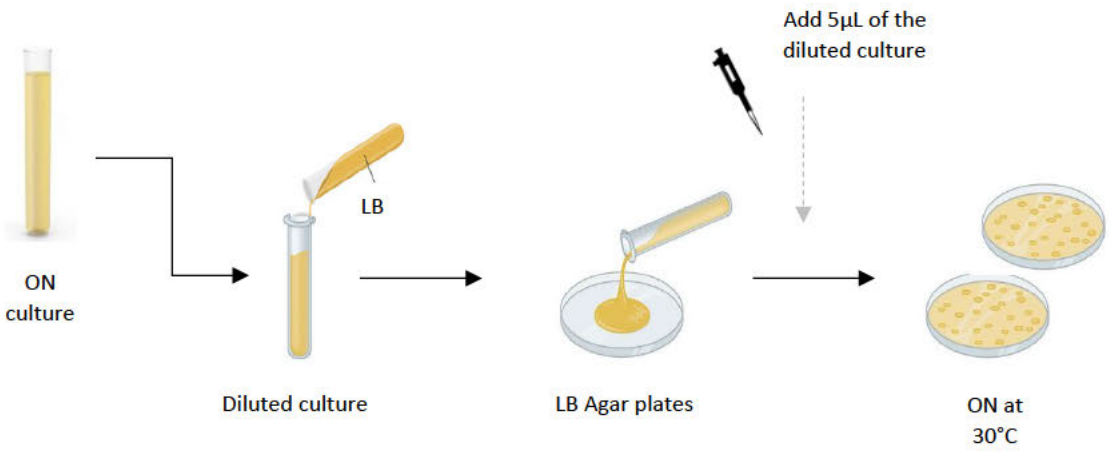
S2.1.3 Correlation between CFU and OD

To obtain the correlation between colony forming units (CFU) and optical density (OD), a Vibrio inoculum was grown for an ON at 30°C with shake (200 rpm), i.e., stock culture. From this culture, serial dilutions were carried, i.e., $1/10^{-2}$ to $1/10^{-6}$ dilutions with LB media. The OD of these diluted cultures was measured to be able to carry the correlation afterwards.

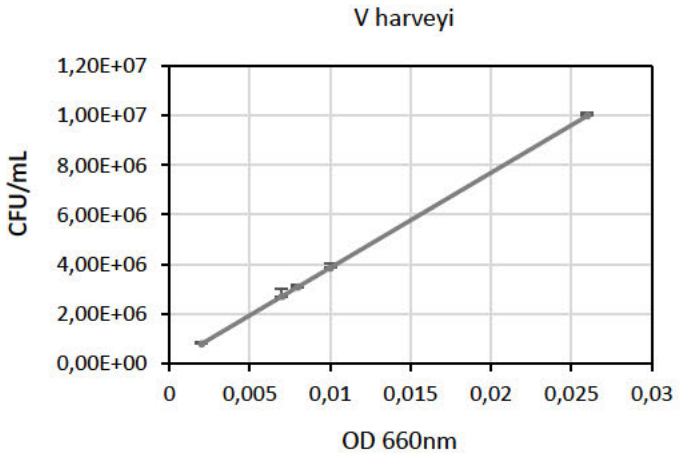
From these dilutions, a predefined amount was taken and was plated in LB Agar plates, in this case, we used 5µL volumes. These LB Agar plates were grown for 24 hours at 30°C to then count the number of colonies the following day.

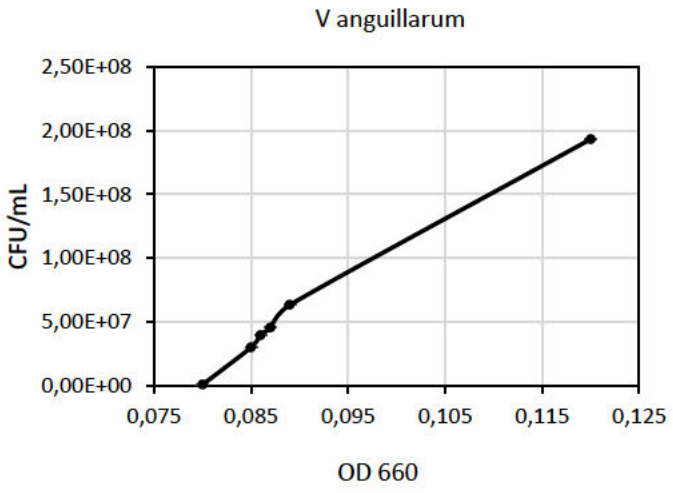
Triplicates of each dilution were carried. Plates with more than 250 CFUs are considered too numerous to count or may inhibit the growth of the neighboring bacteria. On the other hand, plates with less than 25 colonies do not have enough colonies to consider the plate statistically representative[301]. Figure S2.2 shows a schematic representation of the process and the calibration curve for *V harveyi* and *V anguillarum*.

a



b





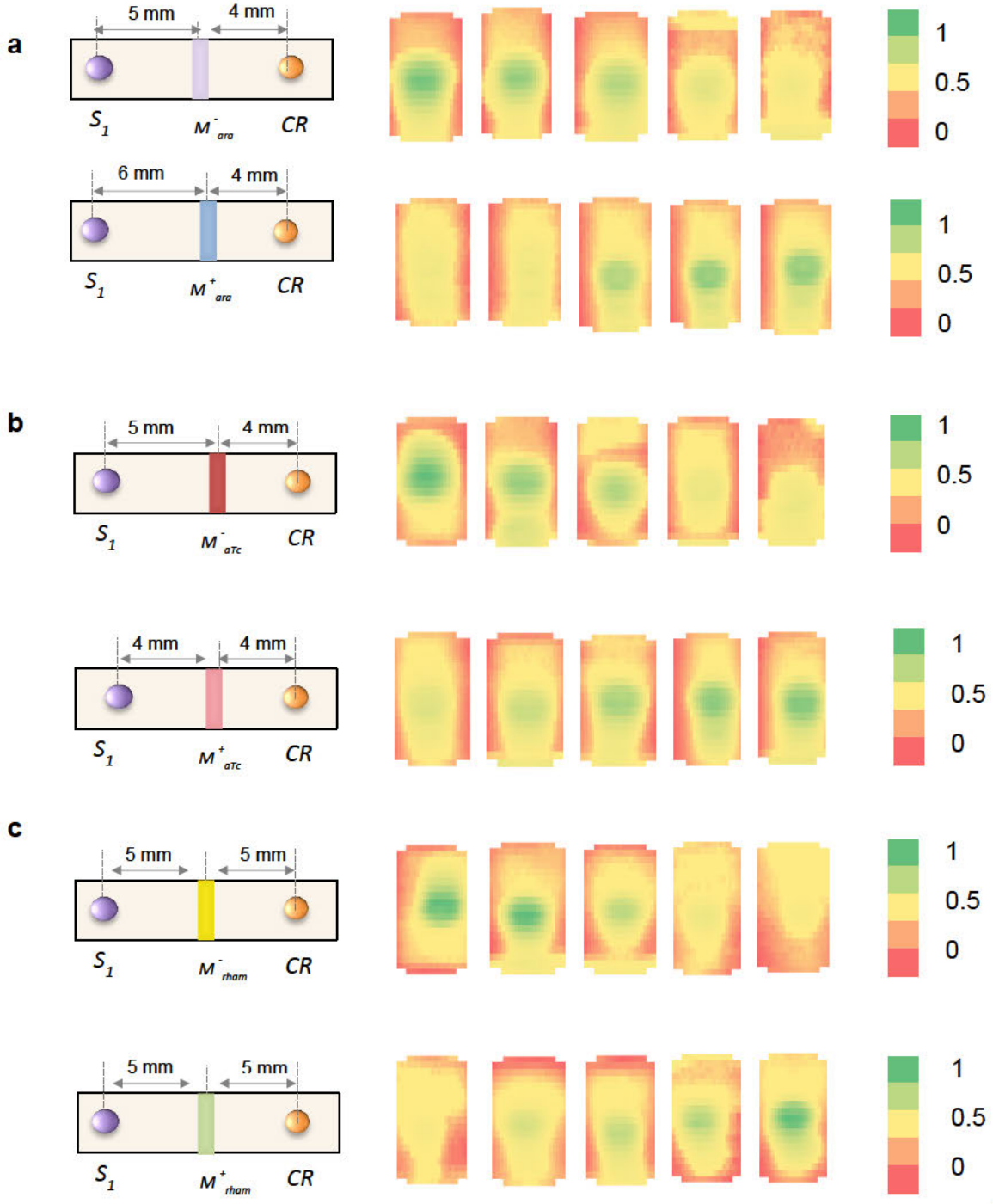
Supplementary Figure S2.2. Correlation between CFU and OD. a. Schematic representation of the process to prepare each dilution from an overnight culture for colony counting. **b.** Experimental results. Both *Vibrio* strains were calibrated to obtain their correlation between cell density and colony forming units. In both figures, the colony count is displayed on the y-axis and the optical density in the x-axis.

ANNEX 2

S3. Chapter 2

S3.1 Additional measures

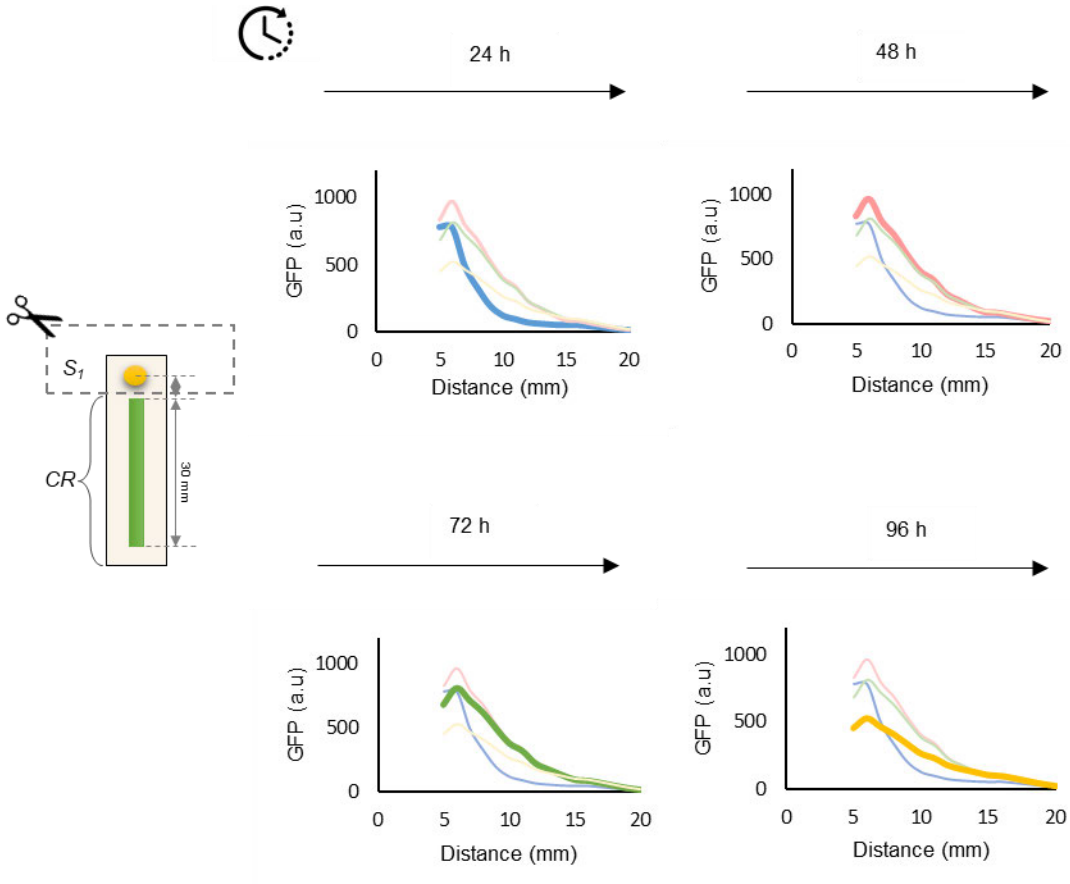
The raw data from figure 2.11 obtained from the intensity measured by scanning our paper strips is depictured in the following figure (Fig.S3.1). Data is displayed for all the negative and positive modulators with their corresponding external inducers, i.e., arabinose, aTc and rhamnose.



Supplementary Figure S3.1. Characterization of different modultatory cells. a. Normalized GFP for positive (M^{+}_{ara}) and negative (M^{-}_{ara}) modulations in response to arabinose. **b**. Normalized GFP for positive (M^{+}_{aTc}) and negative (M^{-}_{aTc}) modulations in response to aTc. **c**. Normalized GFP for positive (M^{+}_{rham}) and negative (M^{-}_{rham}) modulations in response to rhamnose.

Finally, we wanted to assess GFP stability to check whether synthetic circuits would be stable on time. This was relevant for future applications, knowing if the time-window for results visualization was short or long.

For it, we prepared the same experimental setup as figure 2.8 and we removed the supplier cells after the first measure, i.e., after 24 hours. Then, every 24 hours we were measuring the system response (Fig.S3.2)



Supplementary Figure S3.2. Cellular response stability. In this experiment, we withdraw the supplier cells after the first measure to assess if GFP levels were stable during long periods of time.

We could observe some variability between 24h to 48h due to de potential lactone remanent but the response was quite stable, after starting to be diminished after 96 hours. These experiments were telling us that these systems can be quite stable along certain periods of time.

S3.2 Tables with the genetic architecture of the circuit elements

Cell type	Genetic Structure	Strain
S1	B0014 + PTET + B0032 + C0061 B0014 + J23105 + B0032 + E1010 + B0014	Top10
S2	B0014 + I0050 + B0032 + C0061 B0014 + J23105 + B0032 + E1010 + B0014	Top10
S3	B0014 + K914003 + B0032 + E1010 + B0014 B0014 + J23105 + B0032 + E1010 + B0014	Top10
M^{+}_{aTc}	B0014 + R0040 + B0032 + C0060 B0014 + J23105 + B0032 + E1010 + B0014	Zn1
M^{+}_{ara}	B0014 + I0050 + B0032 + C0060 B0014 + J23105 + B0032 + E1010 + B0014	Top10
M^{-}_{aTc}	B0014 + R0040 + B0032 + C0012 + B0014+ R0011 + B0032 + C0060 B0014 + J23105 + B0032 + E1010 + B0014	Zn1
M^{-}_{ara}	B0014 + I0050 + B0032+ C0012 + B0014+ R0011 + B0032 + C0060 B0014 + J23105 + B0032 + E1010 + B0014	Top10
CA	B0014 + J23100 + B0033 + C0061 + B0014 + R0061 + B0033 + C0061 B0014 + J23105 + B0032 + E1010 + B0014	Top10
CR	B0014 + J23100 + B0034 + C0061 + B0014 + R0061 + B0033 + E0040 B0014 + J23105 + B0032 + E1010 + B0014	Top10

Supplementary Table S3.1. Genetic parts involved in each engineered cell strain. Genetic parts were obtained from the Registry of Standard Biological Parts (http://parts.igem.org/).

Part	DNA Sequence
C0061	atgactataatgataaaaaaatcggatttttggcaattccatcggaggagtataaaggtattct aagtcttcgttatcaagtgtttaagcaaagacttgagtgggacttagttgtagaaaataaccttg aatcagatgagtatgataactcaaatgcagaatatatttatgcttgtgatgatactgaaaatgta agtggatgctggcgtttattacctacaacaggtgattatatgctgaaaagtgtttttcctgaattgc ttggtcaacagagtgctcccaaagatcctaatatagtcgaattaagtcgttttgctgtaggtaaa aatagctcaaagataaataactctgctagtgaaattacaatgaaactatttgaagctatatata aacacgctgttagtcaaggtattacagaatatgtaacagtaacatcaacagcaatagagcg atttttaaagcgtattaaagttccttgtcatcgtattggagacaaagaaattcatgtattaggtgat actaaatcggttgtattgtctatgcctattaatgaacagtttaaaaaagcagtcttaaatgctgca aacgacgaaaactacgctttagtagcttaataactctgatagtgctagtgtagatctc
C0060	atgacagtaaagaagctttatttcgtcccagcaggtcgttgtatgttggatcattcgtctgttaata gtacattaacaccaggagaattattagacttaccggtttggtgttatcttttggagactgaagaa ggacctattttagtagatacaggtatgccagaaagtgcagttaataatgaaggtctttttaacgg tacatttgtcgaagggcaggttttaccgaaaatgactgaagaagatagaatcgtgaatatttta aaacgggttggttatgagccggaagaccttctttatattattagttctcacttgcattttgatcatgc aggaggaaatggcgcttttataaatacaccaatcattgtacagcgtgctgaatatgaggcgg cgcagcatagcgaagaatatttgaaagaatgtatattgccgaatttaaactacaaaatcattg aaggtgattatgaagtcgtaccaggagttcaattattgcatacaccaggccatactccagggc atcaatcgctattaattgagacagaaaaatccggtcctgtattattaacgattgatgcatcgtat acgaaagagaattttgaaaatgaagtgccatttgcgggatttgattcagaattagctttatcttca attaaacgtttaaaagaagtggtgatgaaagagaagccgattgttttctttggacatgatatag agcaggaaaggggatgtaaagtgttccctgaatatatagctgcaaacgacgaaaactacg ctttagtagcttaataacgctgatagtgctagtgtagatcgc

R0032	tcacacaggaaag
R0033	tcacacaggac
R0034	aaagaggagaaa
E0040	atgcgtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgtt aatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttac ccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggtt atggtgttcaatgctttgcgagatacccagatcatatgaaacagcatgacttttcaagagtgcc atgcccgaaggttatgtacaggaaagaactatatttttcaaagatgacgggaactacaagac acgtgctgaagtcaagtttgaaggtgataccttgttaatagaatcgagttaaaaggtattgattt taaagaagatggaaacattcttggacacaaattggaatacaactataactcacacaatgtat acatcatggcagacaaacaaaagaatggaatcaaagttaacttcaaaattagacacaaca ttgaagatggaagcgttcaactagcagaccattatcaacaaaatactccaattggcgatggc cctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcccaacg aaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatgg atgaactatacaaataataa
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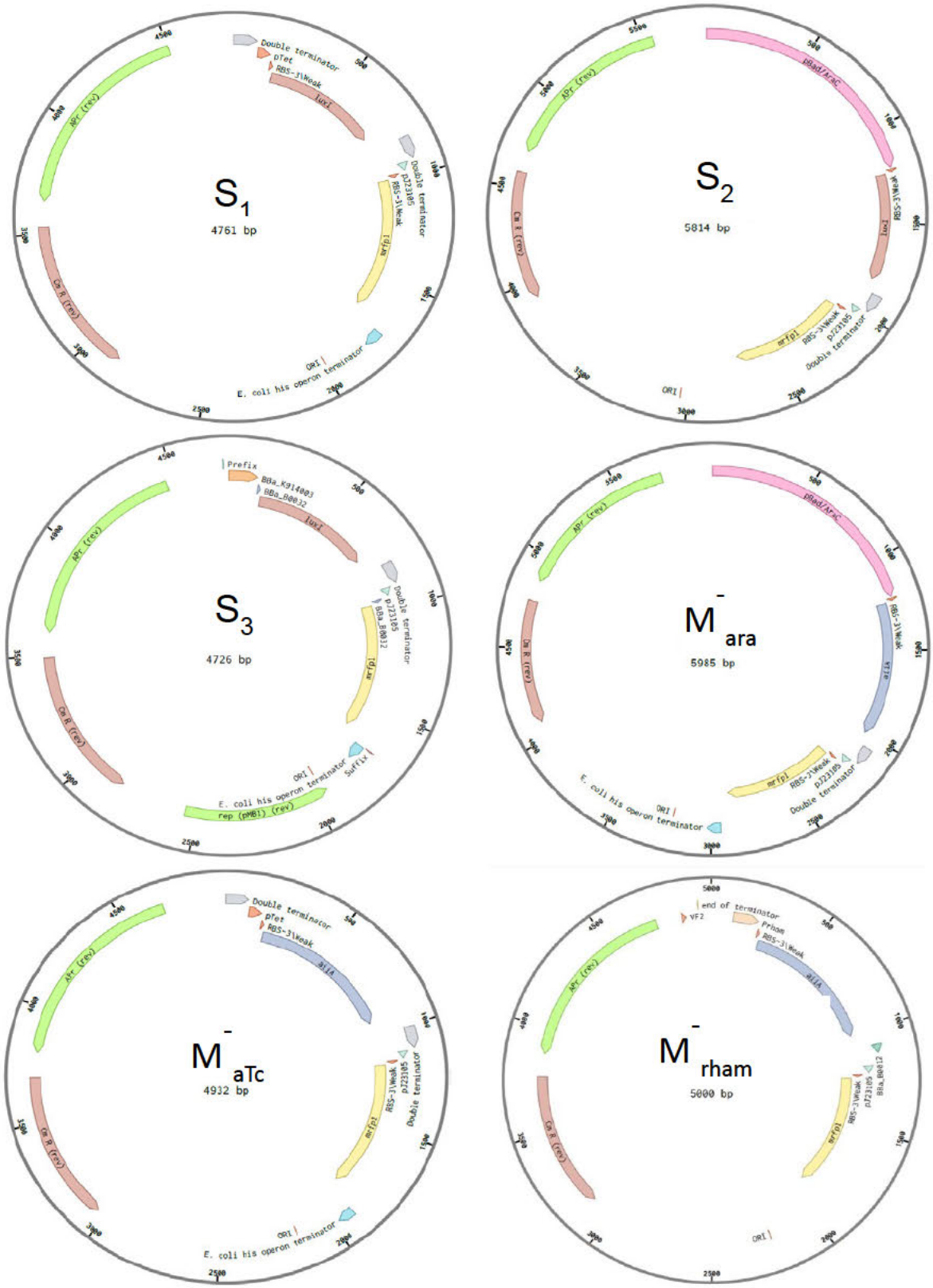
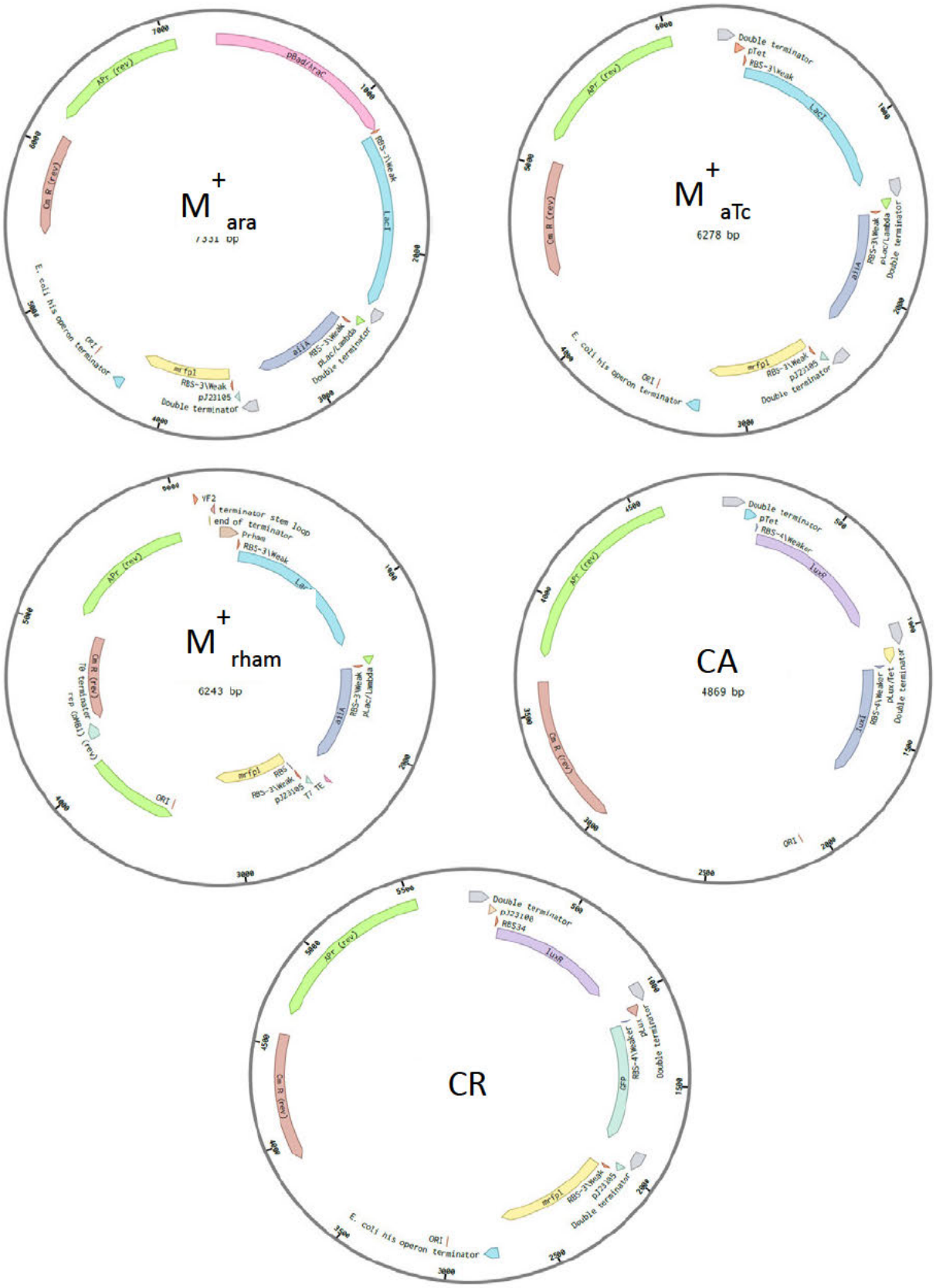
Supplementary Table S3.2. Genetic parts sequence.

S3.3. Plasmid Maps

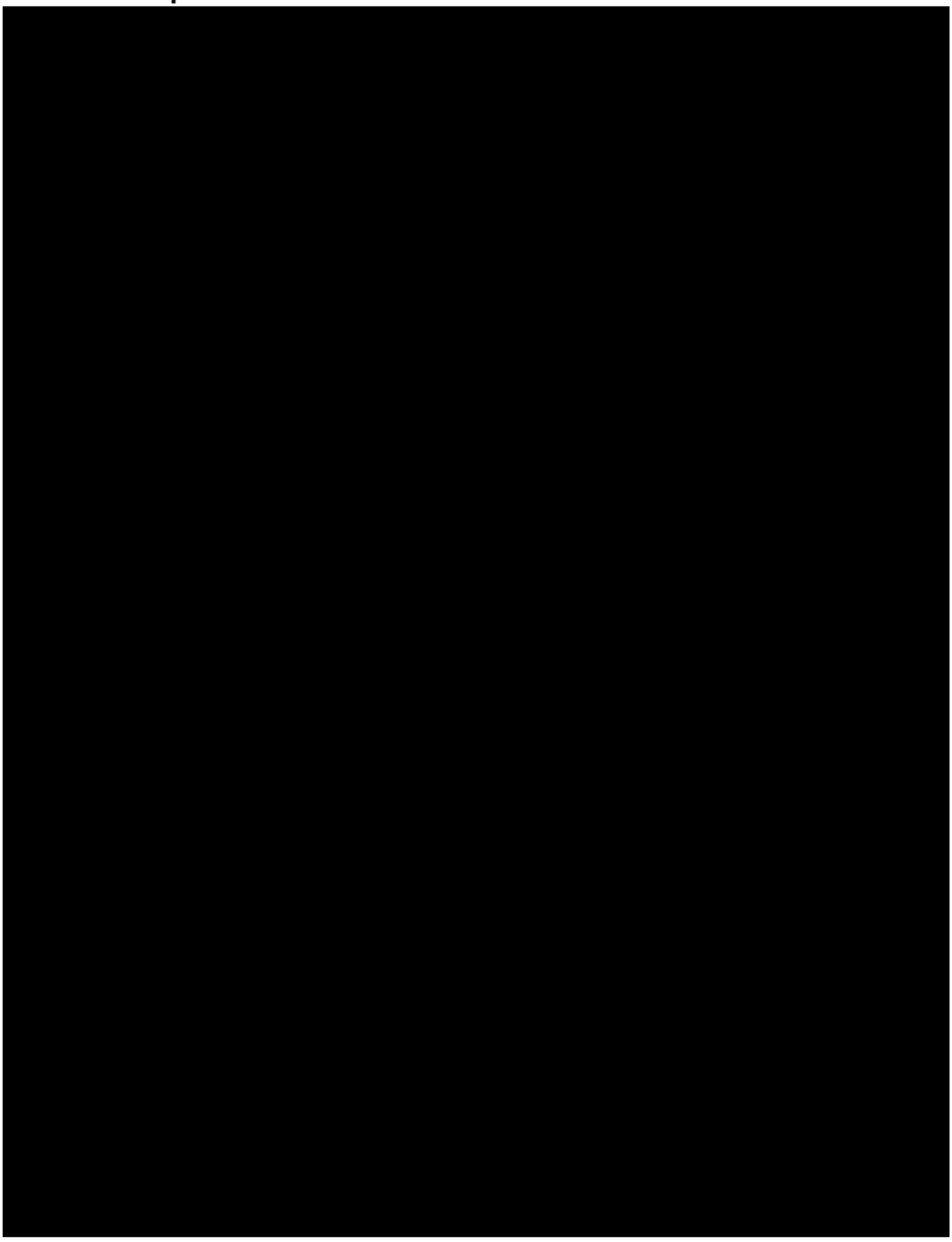
Schematic representation of all genetic constructs.

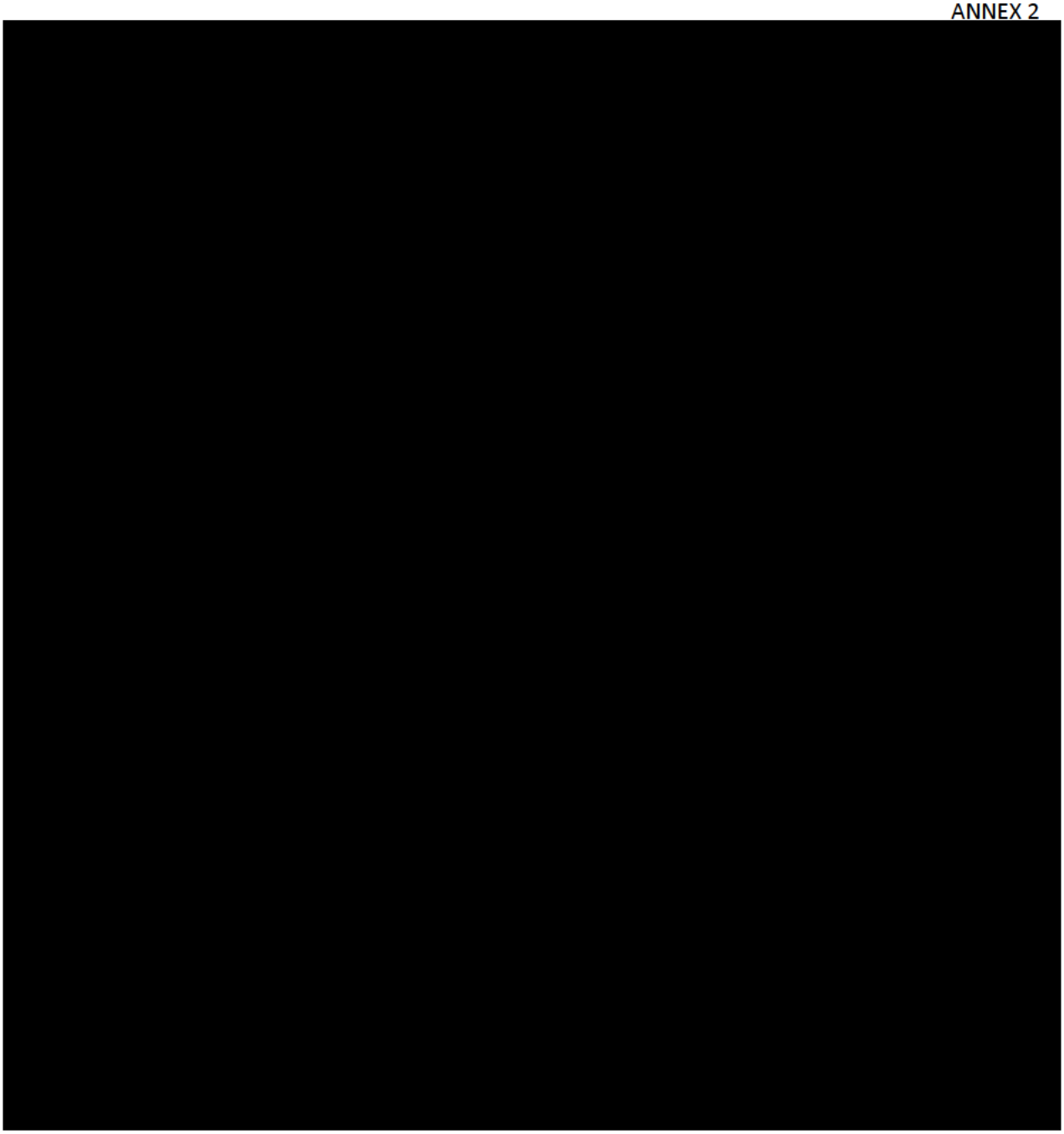


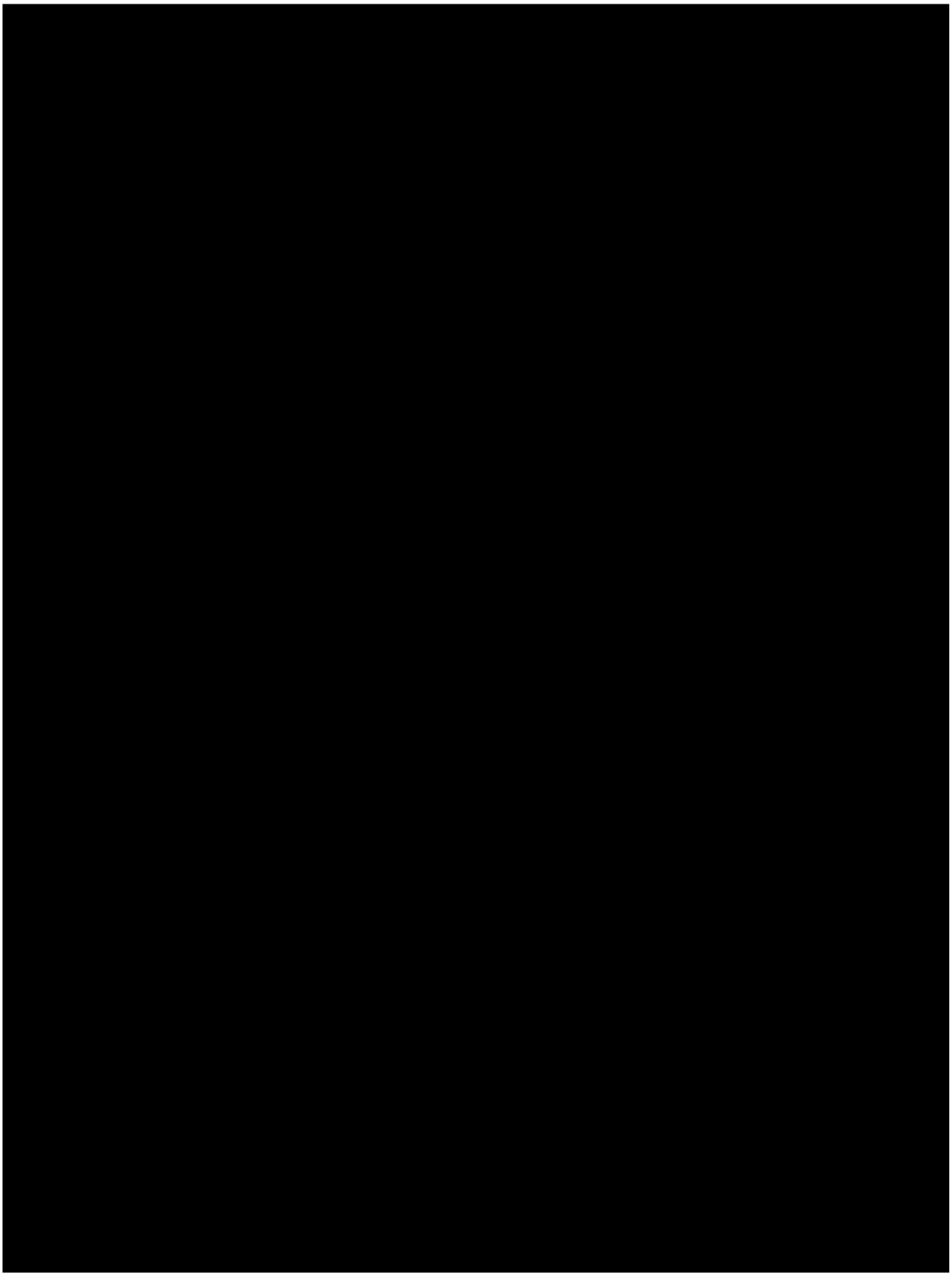


Supplementary Figure S3.3. Plasmid Maps.

S4. Chapter 3







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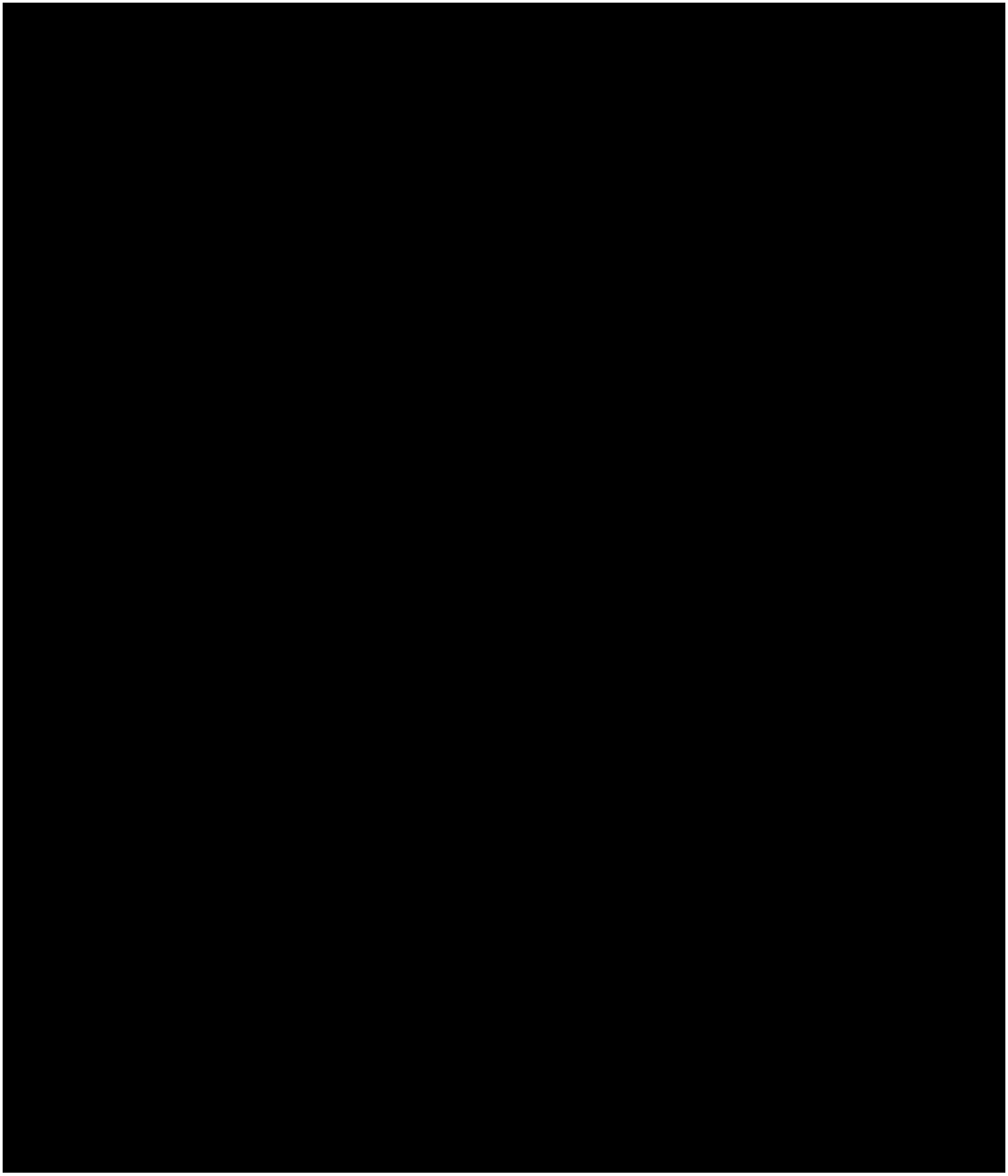

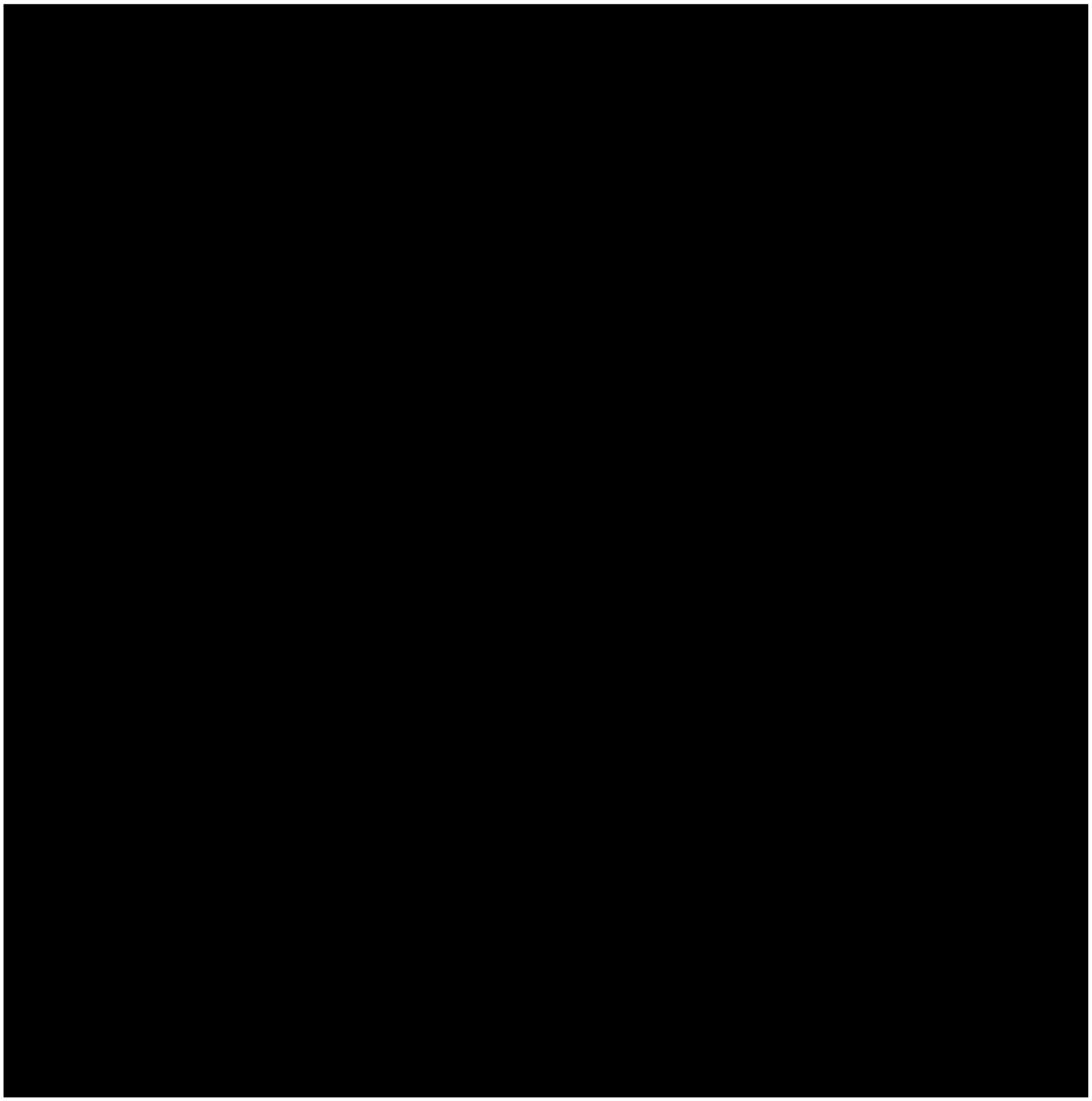

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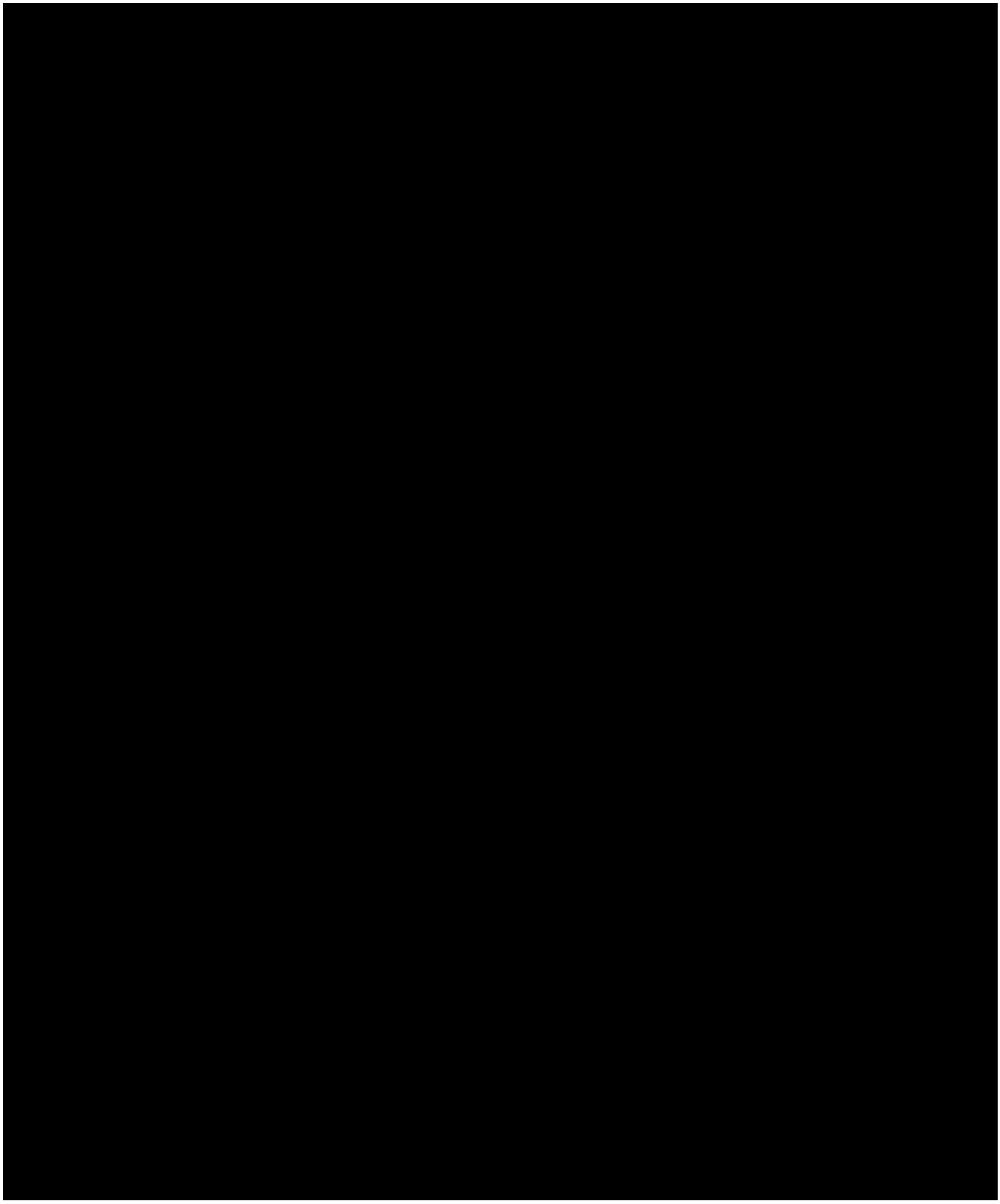

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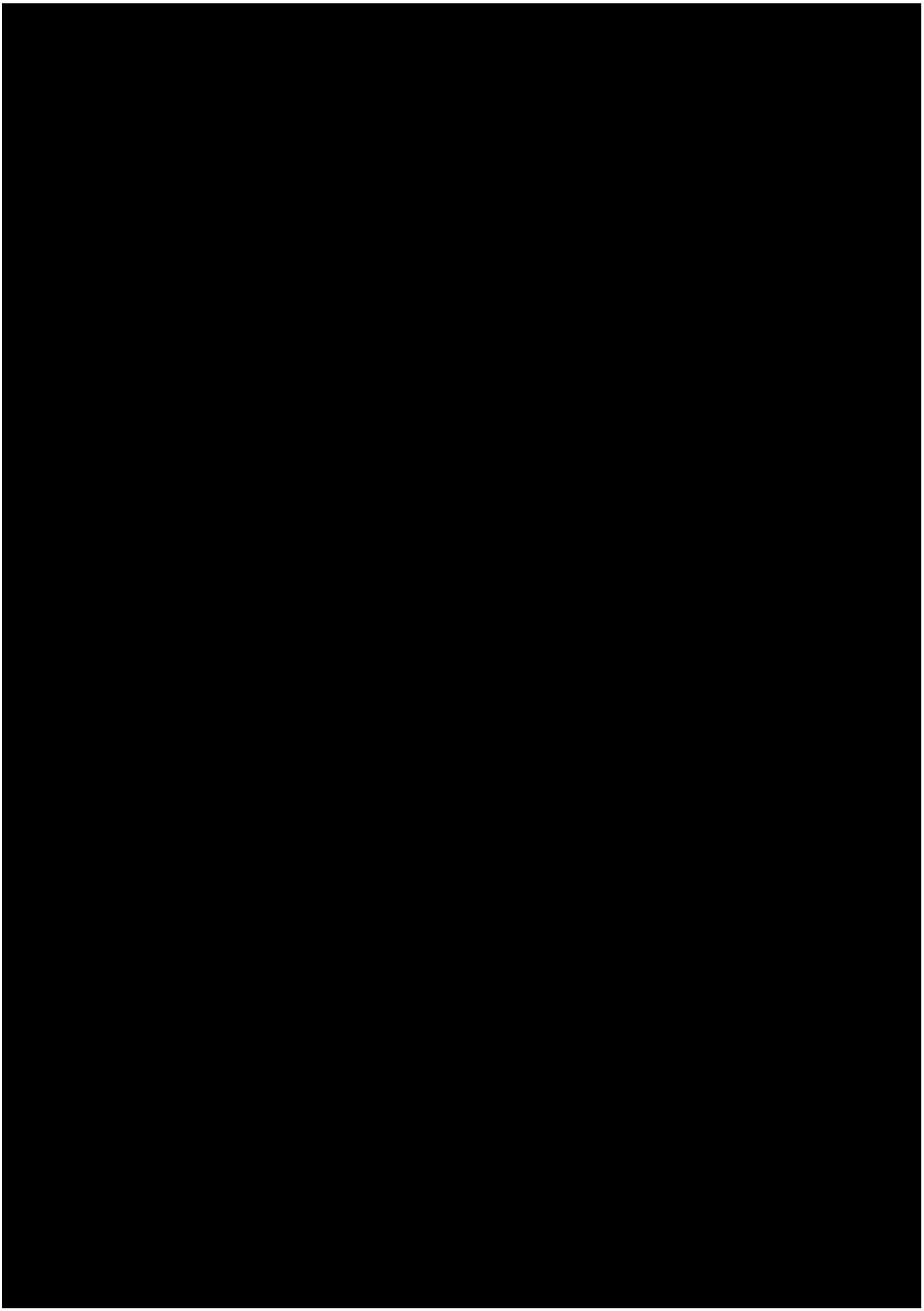

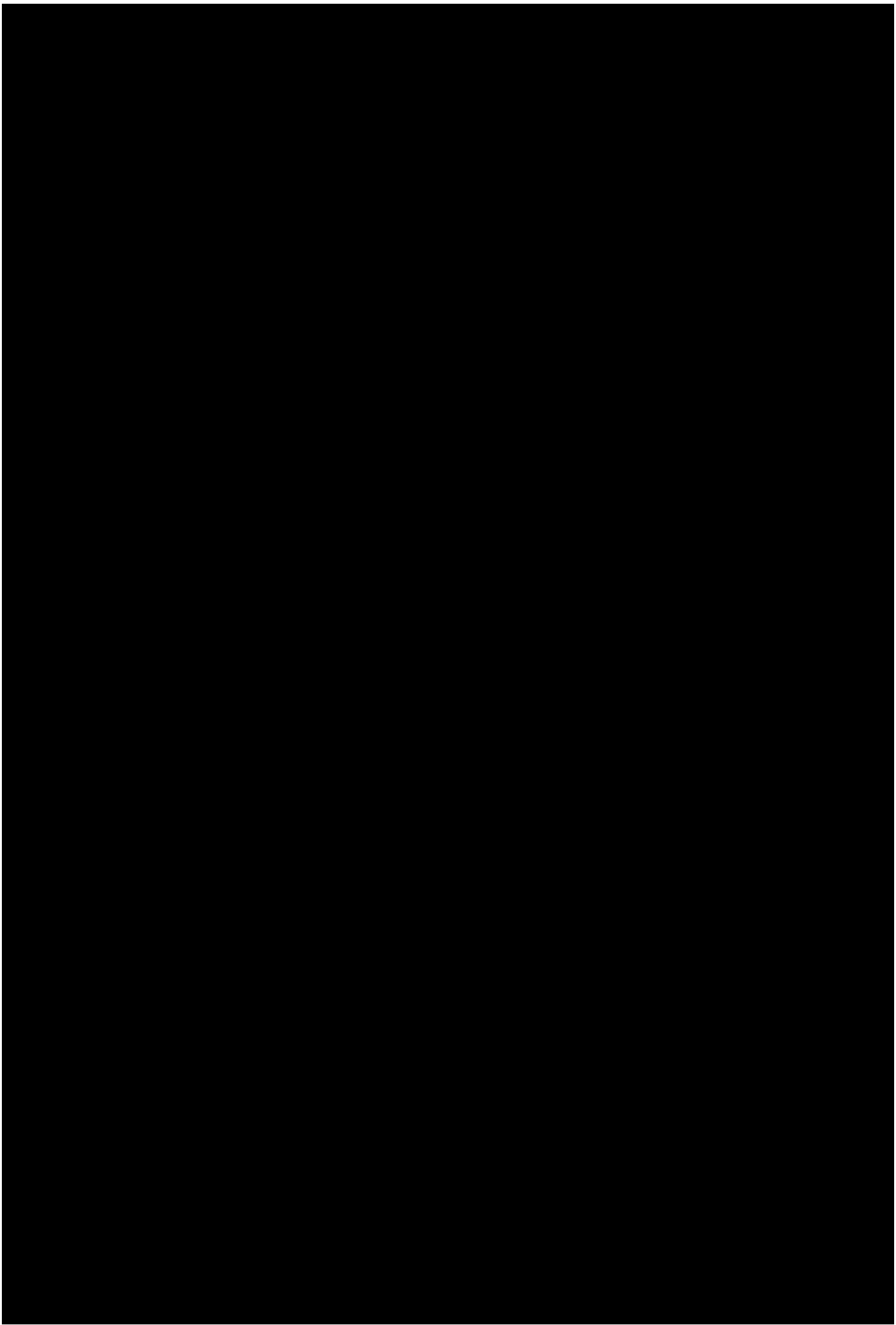

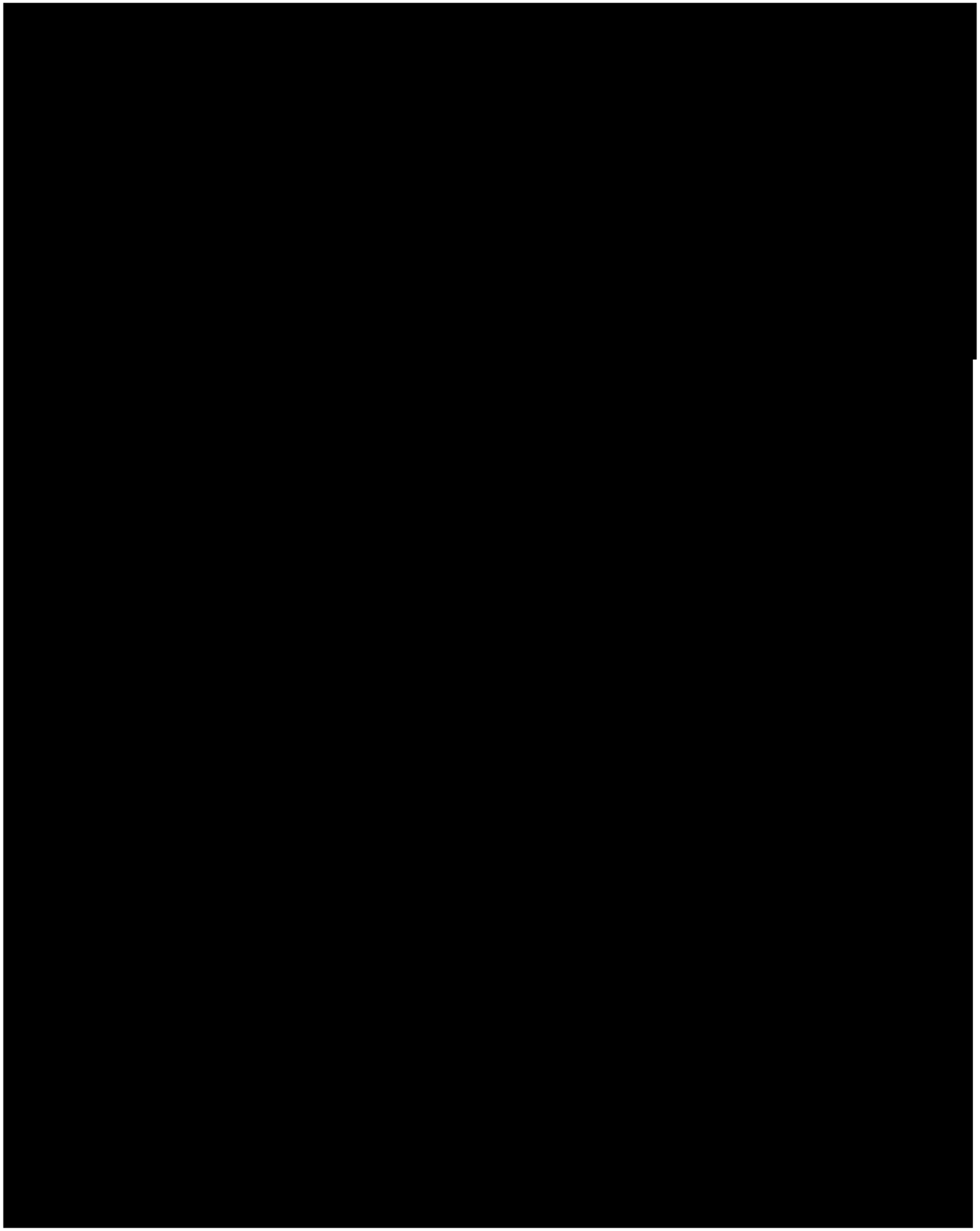

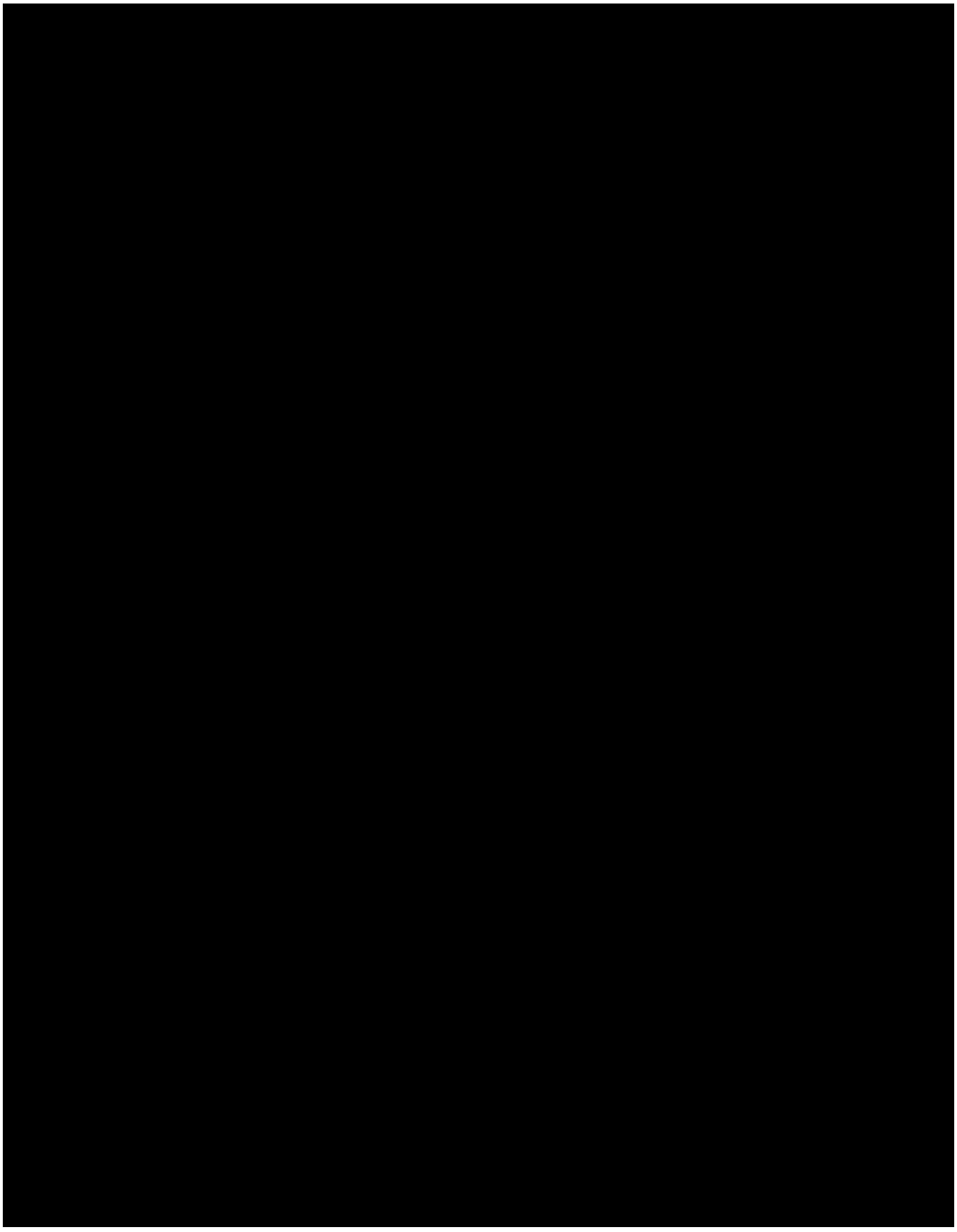

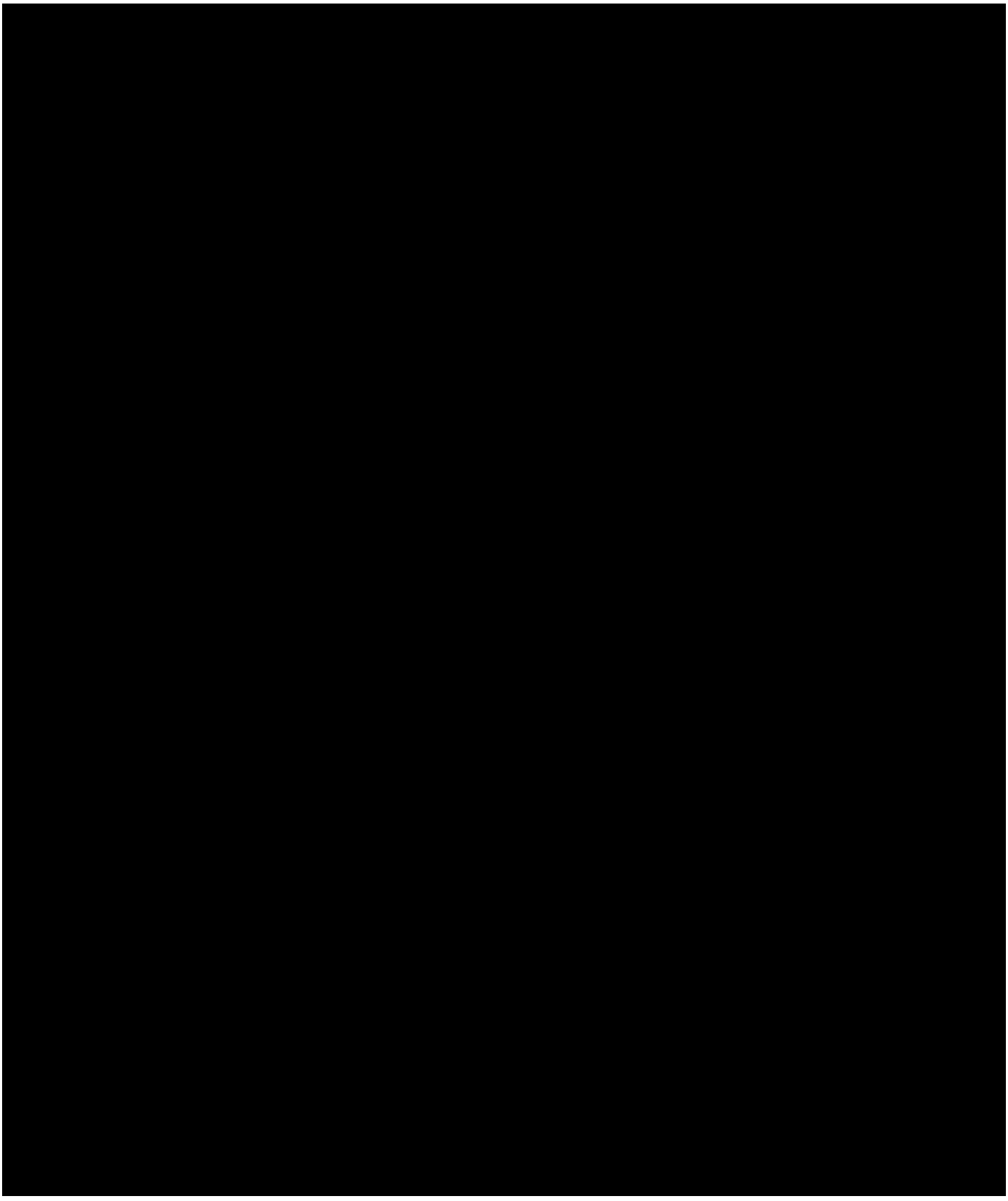

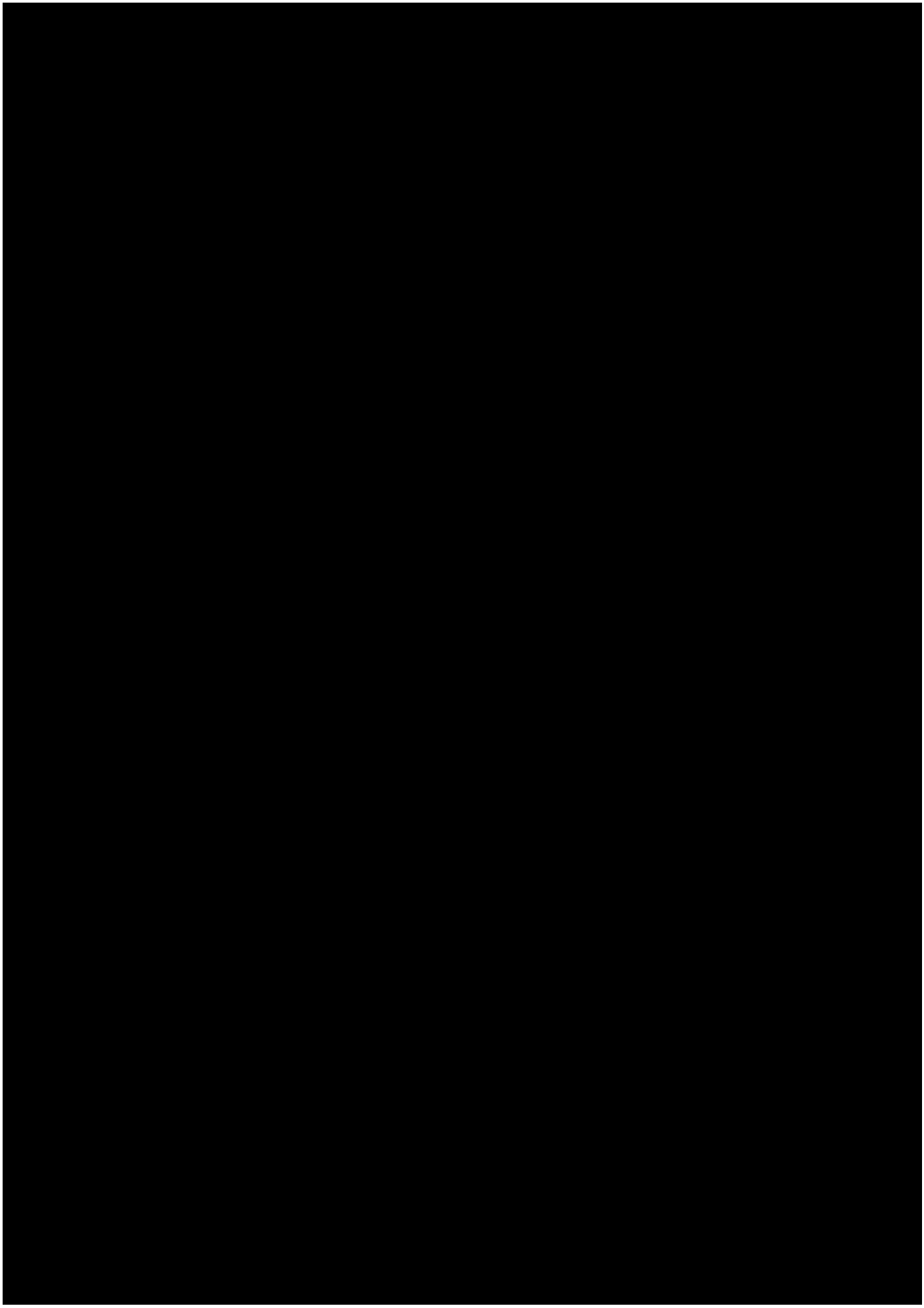

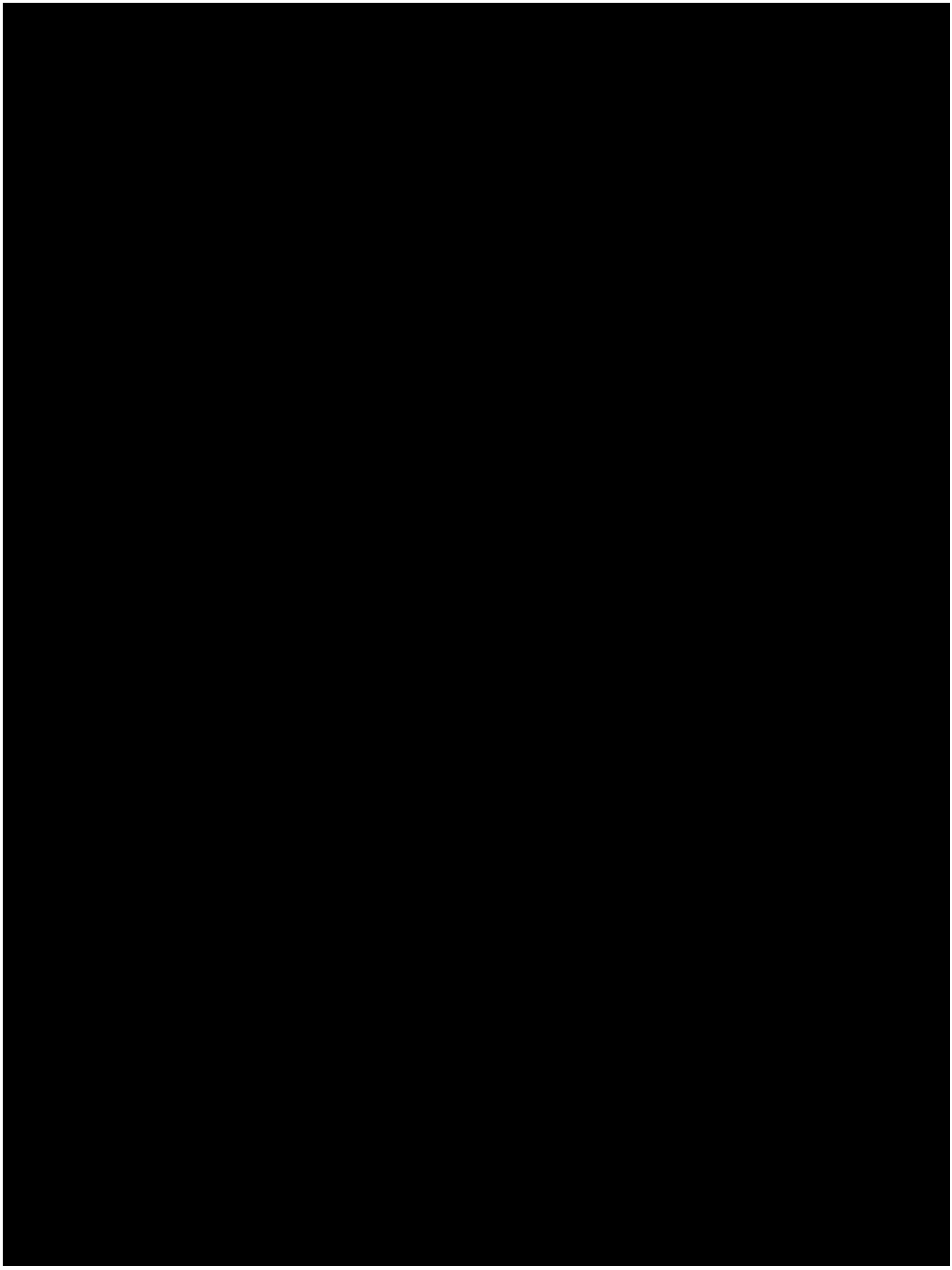

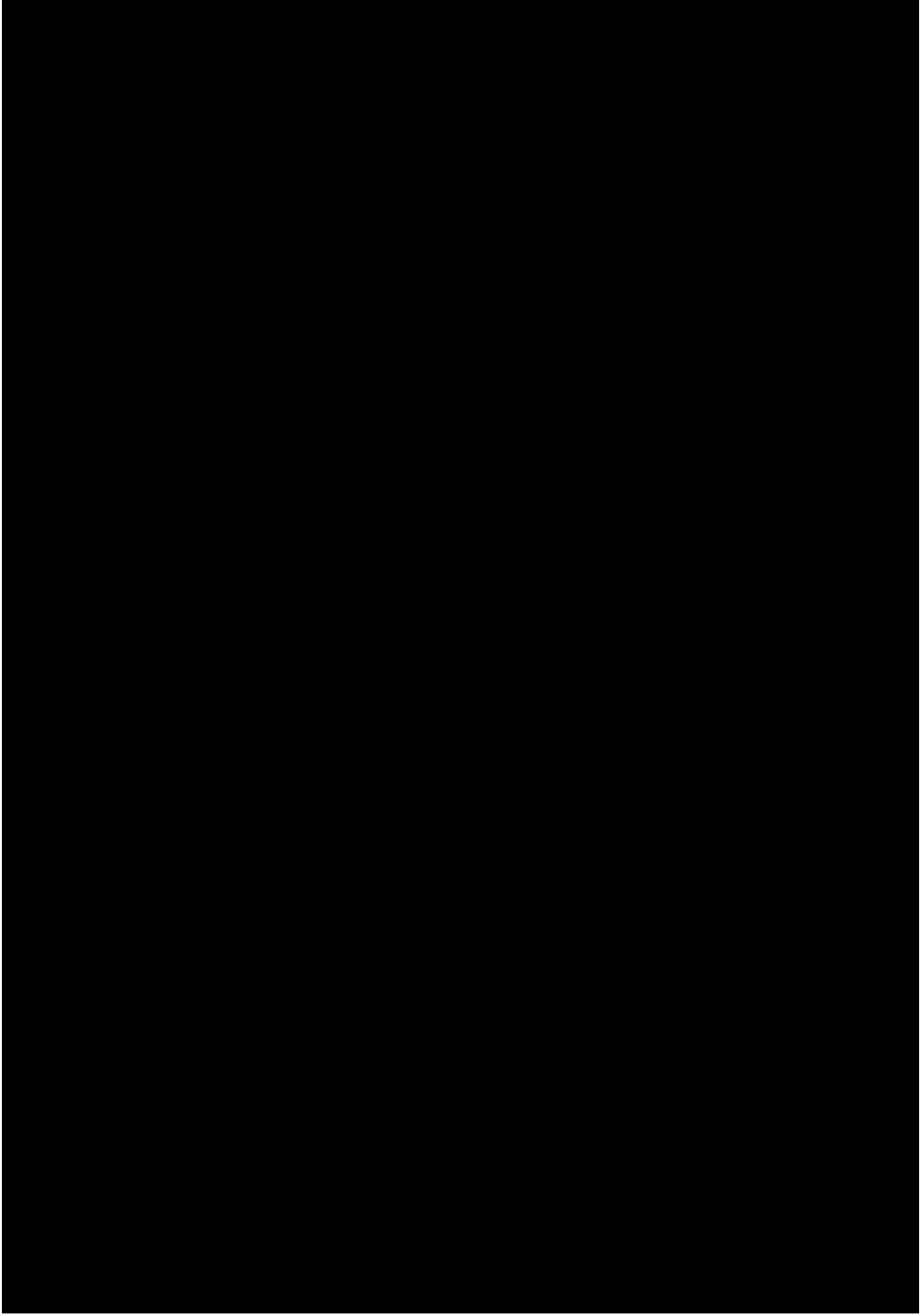

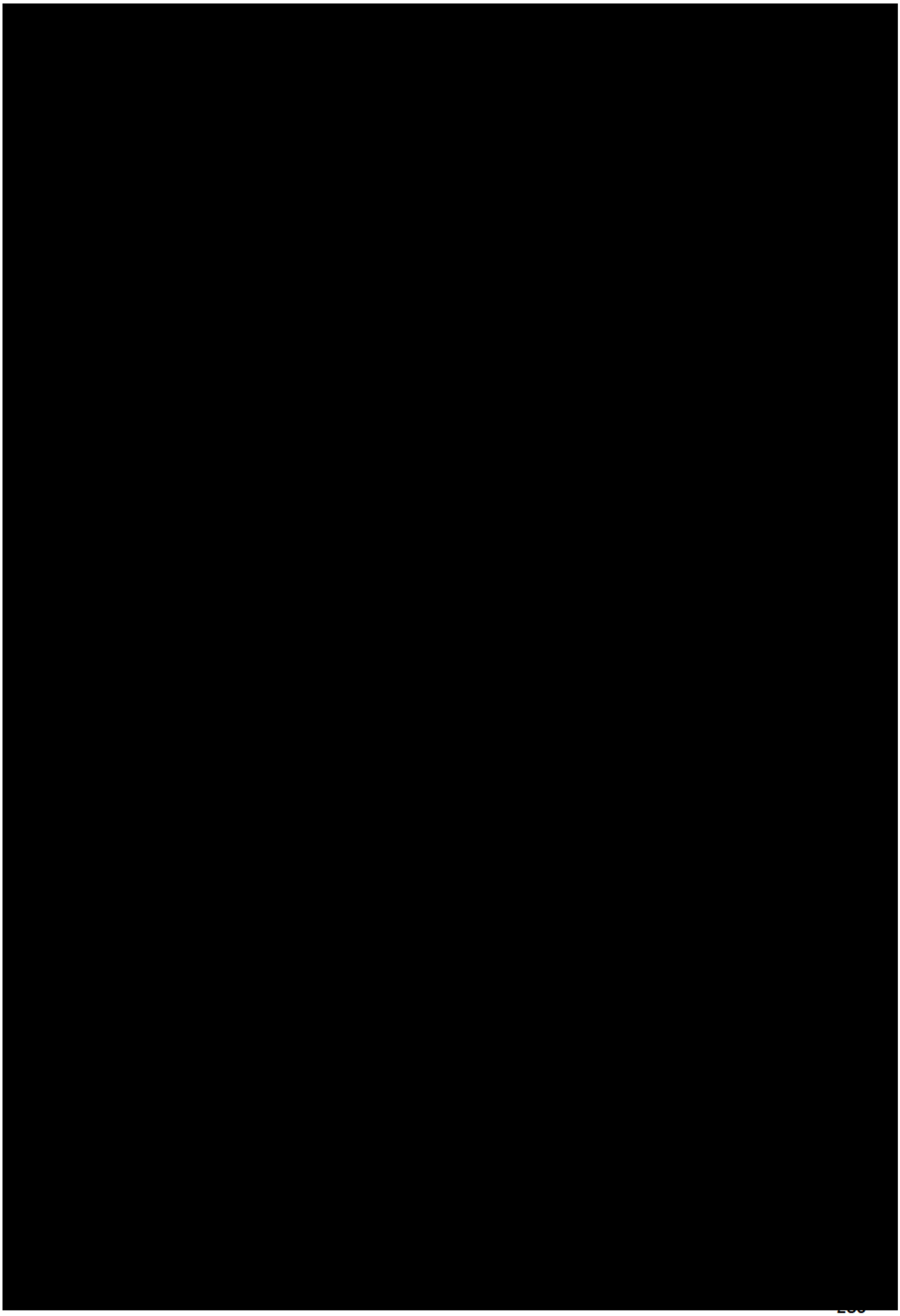

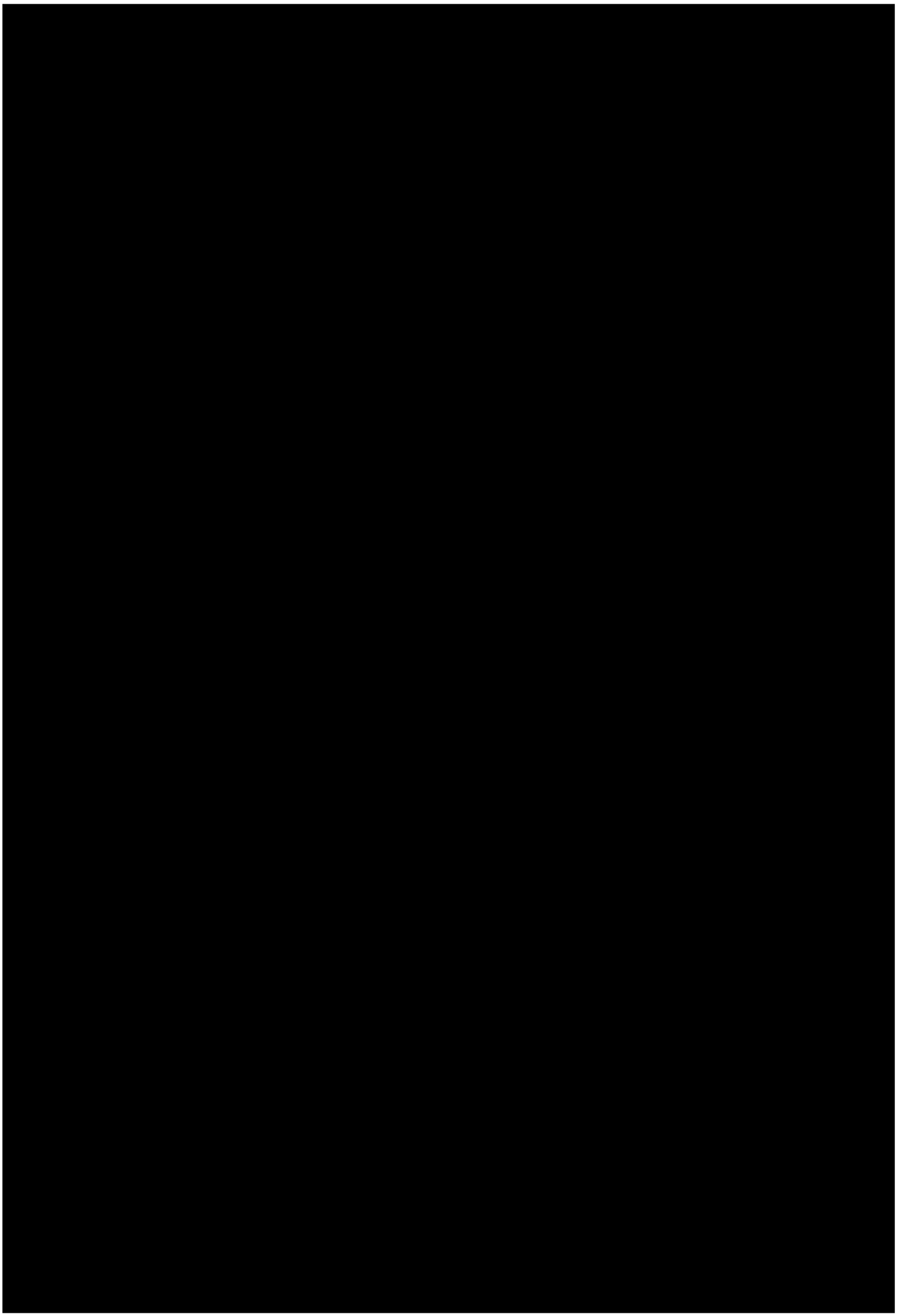

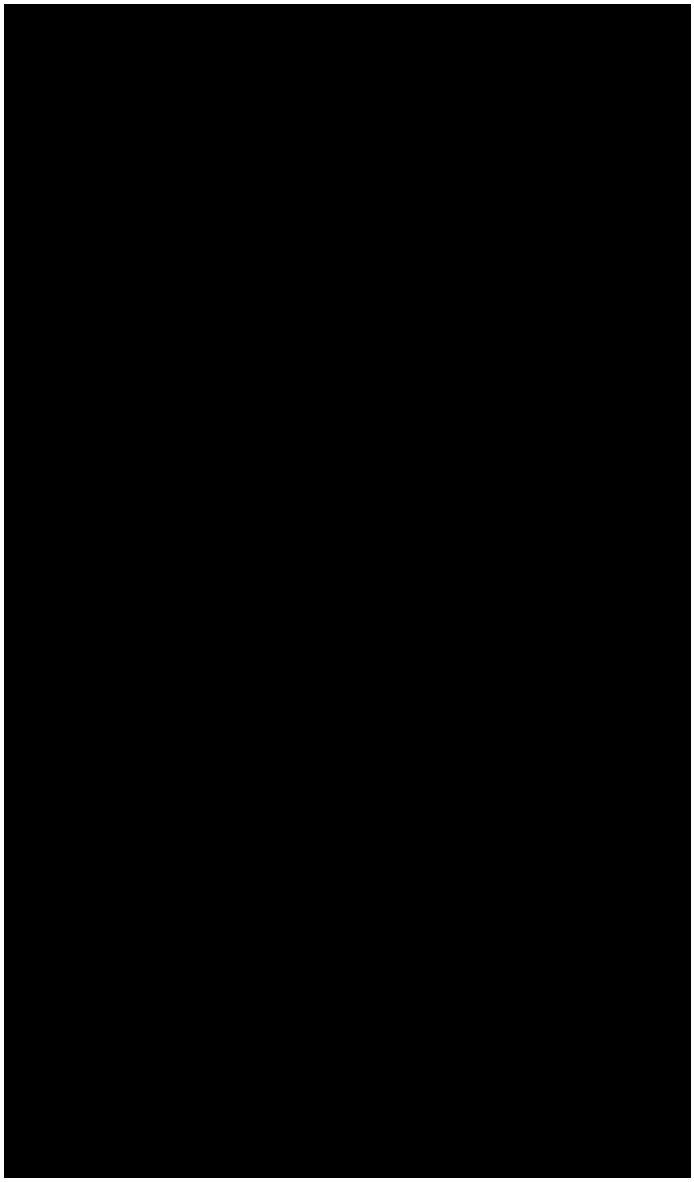

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