Engineering Cellular Communication Systems for Synthetic Biology

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Tiivistelmä – Referat – Abstract

Synthetic biology is an emerging interdisciplinary field of biology that aims to systematically design artificial biological systems. As synthetic biologists seek increasingly complex control over cellular processes to achieve robust and predictable systems. A new frontier in synthetic biology is engineering synthetic microbial consortia. This approach employs the concept of division of labor, instead of introducing large genetic circuitry to homogenous cell populations. In this approach, different cell types are assigned to execute a portion of the overall circuit. Each cell type communicates with their co-worker subpopulations to complete the circuit. The main advantage of this strategy is the reduced metabolic burden on each cell type. Thus, leading to more reliable and stable overall performance. In this work, to simplify cellular communication between the members of the consortium, we used the simple architecture of quorum sensing machinery. We constructed a toolbox that contains promoter, receptor and quorum sensing signal synthase genes along with fluorescent reporters. Using this toolbox, we constructed different cell types that can be used in synthetic consortia forming various communication topologies. We characterized the constructed cell types individually and in co-cultures.

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1. Introduction

Synthetic biology is an emerging interdisciplinary field of biology that aims to systematically design artificial biological systems by applying engineering principles. Synthetic biology has broad applications in key areas such as health, environment and energy. Synthetic biologists are seeking increasingly complex control over cellular processes to achieve robust and predictable systems. In order to accomplish this, synthetic biologists use standardized genetic parts to build biological circuitry. However, on the path to increasing complexity introducing high number of parts is not infinitely feasible. Each synthetic component introduced to the host competes with native cellular processes thus causing a metabolic burden on the cell. This results in unpredictable expression, reduced fitness and even growth defects. One proposed approach to tackle this issue is through the division of labor. In this approach, different cell types are assigned to execute a portion of the overall circuit. Each cell type communicates with their co-worker cells to complete the circuit. The main advantage of this strategy is the low burden on each cell type. Therefore, individual populations perform more reliably and stably. This leads to higher performance of the overall community.(Brenner, You, and Arnold 2008)

As good as the idea of division of labor sounds, designing such systems is challenging. In natural systems that we see the division of labor the balance is perfected with years of evolutionary pressure. Simply because there are too many variables to the equation in design process making the balance and fitness of each subpopulation is highly unpredictable. Composition of the microbiota is affected by the communication between different cell types. The communication topology and mechanisms can be extremely complicated. Additionally, studying such complex systems also requires a unique experimental setup than conventional co-cultures.

In this work, to simplify cellular signaling for both inter and intra-subpopulation communication, we used the simple architecture of quorum sensing. We constructed a tool box that contains promoter, receptor and quorum sensing signal synthase genes along with fluorescent reporters. Using this toolbox, we aimed to construct different multi cellular systems with a bottom-up approach.

1.1. Synthetic Biology

Synthetic biology is an emerging discipline aiming to build increasingly complex novel functional biological systems. Defining this field of biology is challenging since it is still in its infantry. Perhaps the best way to grasp is by comparison. In biotechnology, the process of designing artificial functions has been based on experimental trial and error, therefore time consuming and expensive due to the complexity of biological machinery (Endy 2005). To minimize the complexity, biology research has been focusing on understanding biological elements of systems on individual levels such as genomes, proteins and organisms. Whereas, systems biology, attempts to put the individual pieces revealed by –omics and genome projects in order to have a better understanding of the biological systems as a whole (Pleiss 2006). Like systems biology, synthetic biology focuses on systems with a holistic view but instead of investigating the existing systems synthetic biology aims expand beyond existing with novel assemblies (Andrianantoandro et al. 2006). Synthetic biology aims to build novel biological functions by manipulating and implementing basic standardized elements available to reach complex synthetic systems. An established methodology of rational design of novel synthetic systems that behave as initially predicted could make the design process more efficient thus reducing the time and cost in comparison to conventional biotechnology.

The applied systematic approach of engineering in synthetic biology differentiates it from biotechnology. As other engineering disciplines built around natural sciences established principles can be applied to biology. The most relevant lessons synthetic biologist can get from other engineering disciplines would be abstraction, standardization, and decoupling (Endy, 2005). Abstraction can be considered as having abstract hierarchies where each component in the same level work together and able to convey a desired information to other hierarchical levels. The hierarch in synthetic biology, with increasing level of complexity goes as: DNA, parts, devices and systems. DNA is the basic biological material, at the bottom of the hierarch. Parts perform basic defined functions such as transcription factors, promoters, enzymes. Devices are combination of parts that execute defined functions. Systems consist of several devices. Setting global standards would allow comparability of parts. Standardization is setting the norms to evaluate a process.

Setting a universal reference for enzyme activity is an example to standardization principle. Another existing example from biology is the sequencing quality score, Phred score, is an established measure of quality of sequencing reads thus performance of the sequencing process. Decoupling is separating a complex problem into simpler ones and addressing them. For example, developing the custom DNA synthesis without sequence length limits would decouple design and construction of synthetic circuits. Applying the lessons learned from engineering, developing field of synthetic biology could revolutionize biology research by pushing the limits of trial and error process (Andrianantoandro et al. 2006).

Being able to rationally engineer complex biological systems with novel functions is promising for wide range of applications from biosensors, synthetic pattern formation and therapeutics. Synthetic biologists have designed and constructed synthetic circuits that can make predictable decisions based on certain inputs through designed gene networks that functions as logic gates (Nielsen et al. 2016). Such synthetic circuits can be perform biological computation in a manner that a circuit would take inputs and give an output. For biosensor applications recruiting the capability to interpreting multiple inputs of logic gates is useful. For example, synthetic biologists constructed a biosensor that operates via an AND gate (Anderson, Voigt, and Arkin 2007). An AND gate performs a basic function by returning a positive output only when two positive inputs are given. Another example, a synthetic ecosystem was constructed consisting two populations of E.coli mimicking the dynamics of predator-prey ecosystem by bi-directional signaling (Balagaddé et al. 2008). This study shows that synthetic biology can also offer novel methods to study the intricate biological interaction by simply re-designing it. One of the most impressive achievements of synthetic biologist is the production of anti-malaria drug precursor in yeast by pathway engineering (Ro et al. 2006). Considering the wide range of applications, synthetic biology holds a great potential, yet complexity of biological systems is an obstacle in the way of development of the field.

1.2. Limitations of Synthetic Biology

Despite the potential of the field, engineers of synthetic biological systems are facing several problems. Unlike mechanical engineers who has a set of standard tools developed

for the field, biologists still lack established standard accessible tools. For synthetic biologists the most basic toolbox would be a vast array of standardized parts. Such synthetic biology toolbox would contain standardized parts such as promoters, transcription factors, enzymes, proteins. There has been a collective effort to put together a standard parts registry initiated by the International Genetically Engineered Machine (iGEM) Foundation. Yet, there is still limited number of well-characterized parts. Another concern for synthetic biologist is the metabolic burden of additional parts on the expression host. This is especially problematic for devices that contain high number of parts. An engineered biological device's function is dependent on its host's ability to execute since it will consume the resources of the host. For example, level of a recombinant protein expressed is dependent on the available resources of the expression host such as RNA polymerase, ribosome, tRNA. Metabolic burden is a limiting factor for scalability of synthetic circuits. (Borkowski et al. 2016). Another limitation is the possible undesired interaction of elements used in a synthetic circuit itself. A simple example of this is regulatory elements. Such elements can be affected by the neighboring sequences (Rudge et al. 2016). All these contributing factors limit our ability to forecast the behavior of a synthetic circuit.

1.3. Division of Labor

One approach that can tackle issues faced in synthetic biology is division of labor. The principle is to divide large complex biological circuits into specialized subpopulations and use them in combination. With this approach, smaller circuits divided in different subpopulations would allow usage of the same part to be used in multiple subpopulations, thus relieving the issue of limited of available parts. For example, one repressor can be used in many times in a multicellular circuit without interference since the parts are physically separated in different cells. The division also ensures less undesired interactions between different biological parts of large and complex circuits, for example, possible effect of a repressor on an unspecific promoter. The most exciting benefit of the labor division is perhaps the reduced metabolic burden on each subpopulation. This allows more robust and reliable behavior of the expression host therefore provides a better functioning subpopulations and synthetic microbiome. Thus, division of labor allows scala-

bility of biological circuits. Over all, an engineered microbial consortia would have capability of accomplishing more complex tasks compared to a homogeneous culture (Brenner, You, and Arnold 2008).

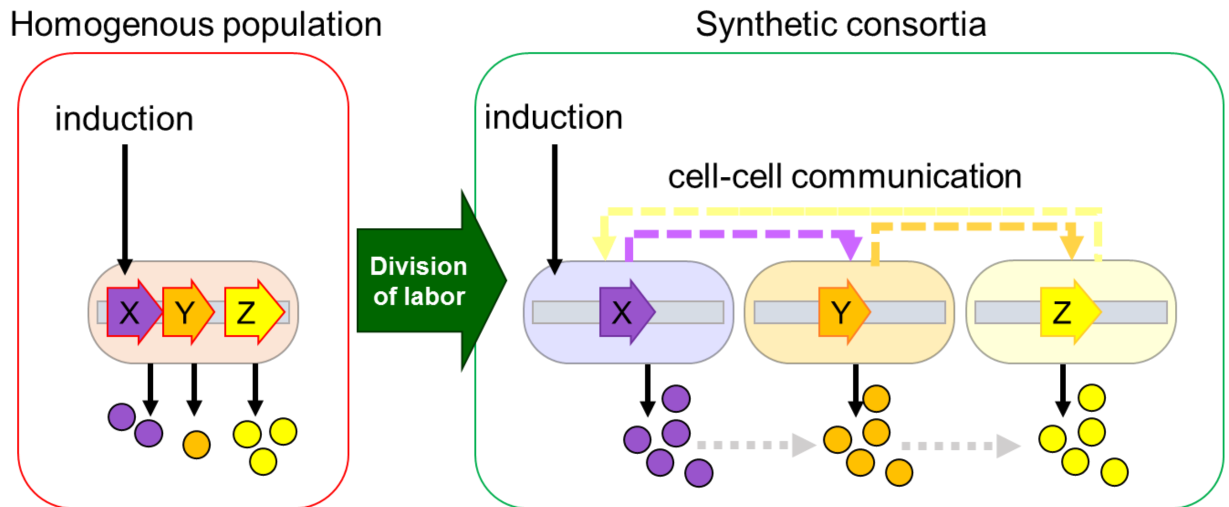


Fig 1. The division of labor concept is illustrated. The cell illustrated in red box expressing all three genes, X, Y and Z. This leads to the expression host a bigger metabolic burden. In the green box, the same genetic load is divided to three different cell types illustrating the division of labor concept. In green box, obtaining the same output as in red box is more robust and predictable.

1.4. Cell-Cell Communication

What distinguishes multicellular organisms from an unorganized cell mass is the ability to undertake complex tasks. This is only possible with specialization and communication thus division of labor. Bacteria have thought to be single, self-sufficient, and simple organisms until the discovery of quorum sensing. However much like multicellular organisms bacteria engage in complex multicellular interactions and react according to received signals (Atkinson and Williams 2009). Over the last 3 decades, research revealed that quorum sensing takes part in vast variety of biological processes from bioluminescence to biofilm formation (Claessen et al. 2014).

1.4.1. Quorum Sensing

Using the ability to communicate with one and other, bacteria can form complex architectures. One of the most notable examples is biofilms. Both formation and maintenance

biofilms require cell-cell communication. Biofilms can host different species that live together in common protective matrix. Members of the bacterial architecture retain various interactions among different species such as predator-prey relationship. They even learned how to cheat by not contributing the communal tasks but benefiting from communal goods. (Dunny, Brickman, and Dworkin 2008). Bacteria can participate in such highly complex coordinated behavior because they can accomplish cell-cell communication.

Quorum sensing is a cell-cell communication mechanism that enable bacteria to alter behavior in a collective manner by regulating gene expression depending on the cell density and composition (Papenfort and Bassler 2016). Quorum sensing is first described in 70's, as cell density dependent alterations in bioluminescence behavior of *Vibrio fischeri*, which regulates the expression of a luciferase enzyme. (Nealson, Platt, and Hastings 1970) *Vibrio fischeri* lives in a symbiotic relation with some marine animals where the host provides the bacterium with nutrient rich niche in return *Vibrio fischeri* provides light to the host via luciferase enzyme (Miller and Bassler 2001; Ruby 1996). For the host light is a useful for attracting prey, defending against (or hiding from) predators and mating (Morin et al. 1975). Study of bioluminescent bacteria *Vibrio fischeri* revealed that no bioluminesce is observed in freshly inoculated cell cultures. However, as the culture reached to higher densities, in late-exponential phase, a rapid burst of bioluminescence was observed (Nealson, Platt, and Hastings 1970). This phenomenon is referred as autoinduction since there was no externally added compound to induce the bioluminescence. This phenomenon is now known as quorum sensing, the term is suggested in 1994 to avoid the confusion between autoinduction and autoregulation (Fuqua, Winans, and Greenberg 1994; Turovskiy et al. 2007). Quorum sensing follows a general mechanism of action. As the population density increases, diffusible signaling molecules (also referred as autoinducers) that are produced in basal levels by each cell accumulates. When concentration of autoinducer molecules in the surrounding exceeds a threshold, it interacts with its cognate receptor partner. Interaction of an autoinducer molecule and its cognate receptor enables activation of certain gene cascades (Atkinson and Williams 2009). Usually, such quorum sensing activated cascades control mechanisms that are effective when carried out collectively (Bassler and Losick 2006).

1.4.2. Mechanism of Homoserine Lactone Mediated Quorum Sensing

Two proteins play role in regulation of bioluminescence of *Vibrio fischeri*, LuxI and LuxR (Engebrecht and Silverman 1984). LuxI protein (gene product of *luxI* gene) is an acyl homoserine lactone synthase. Synthesis of quorum sensing autoinducer, N-3-(oxo-hexanoyl)-homoserine lactone (3OC6HSL), is directed by LuxI enzyme (Eberhard et al. 1981). S-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) are substrates of LuxI (A. L. Schaefer et al. 1996). LuxR is the cognate receptor of 3OC6HSL, autoinducer synthesized by LuxI. At high cell density conditions, accumulated 3OC6HSL binds to LuxR. LuxR bound with its cognate autoinducer acts as a transcription activator by binding to lux box controlling bioluminescence operon (*luxICDABE*) (Ng and Bassler 2015).

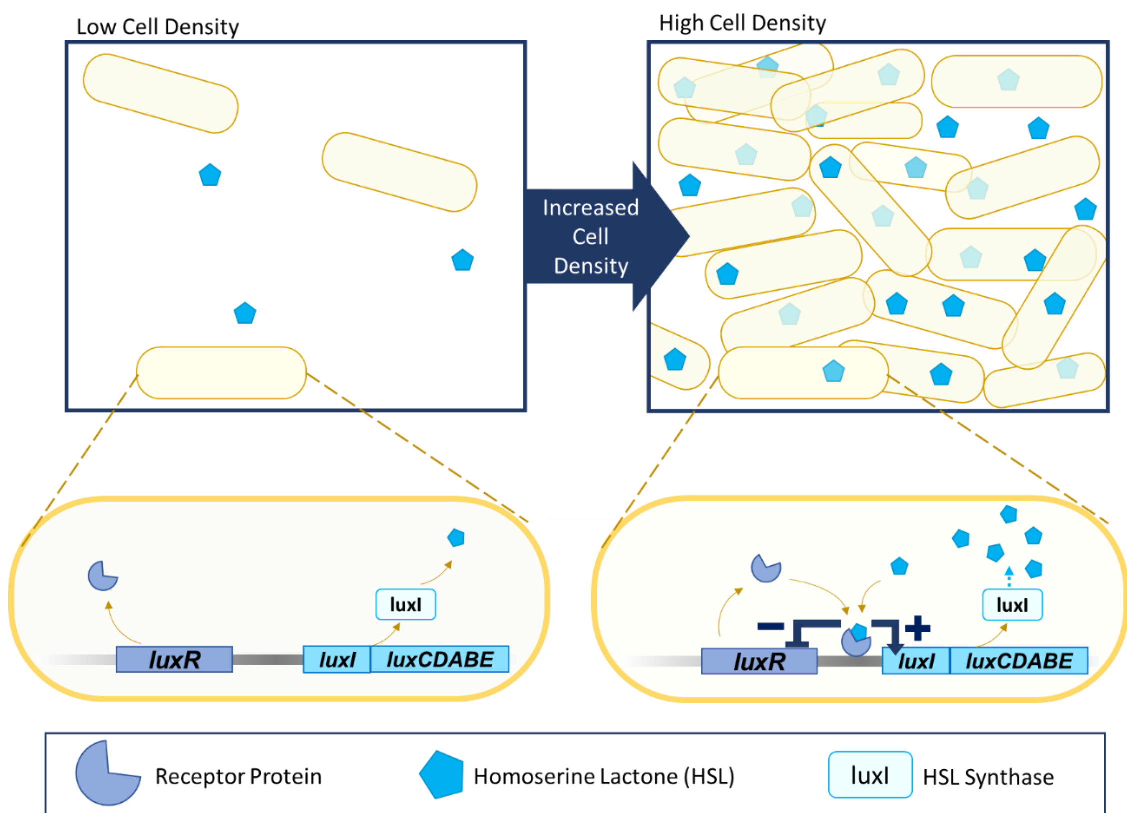


Fig 2. Illustration of density dependent behavior via quorum sensing in *Vibrio fischeri*. *LuxI* gene encodes for LuxI protein that synthesize Lux-HSL. Lux-HSL diffuses freely through the cell. *LuxR* gene encodes for LuxR receptor protein that recognizes Lux-HSL. In low density conditions, basal levels of *luxI* and *luxR* genes expressed. Since HSL levels are low Lux-HSL and its cognate receptor LuxR does not bind. In high density HSL and LuxR binds and acts as transcription factor regulating expression of luxI and luxR genes (negative feedback and positive feedback) and other target genes.

Both gram-positive and gram-negative bacteria use quorum-sensing machinery to regulate their metabolism. Gram positive bacteria utilizes secreted small peptides (autoinducer peptides, AIPs) and a kinase cascade that recognizes autoinducer peptides in high density and starts a phospho-transfer to a response regulator that regulates gene transcription (Reading and Sperandio 2006). In gram-negative bacteria, homologs of LuxIR type quorum sensing system that utilize diffusible homoserine lactones (HSL) as autoinducers-is the most prominent and best-understood quorum sensing mechanism.

Homoserine lactones contain a characteristic homoserine lactone ring and a chain containing 4 to 18 carbons (Papenfort and Bassler 2016) (fig 3). For example, *Rhizobium leguminosarum* uses an acyl-HSL (AHL), N-(3-hydroxy-7-cis-tetradecenoyl)-L-homoserine lactone and LuxR homolog (CinR) (Lithgow et al. 2000) (fig3). Recent studies uncovered that several other signaling molecules such as aryl-homoserine lactones, α pyrones and dialkylresorcinols take part in quorum sensing mechanism other than AHL’s (Brameyer, Bode, and Heermann 2015).

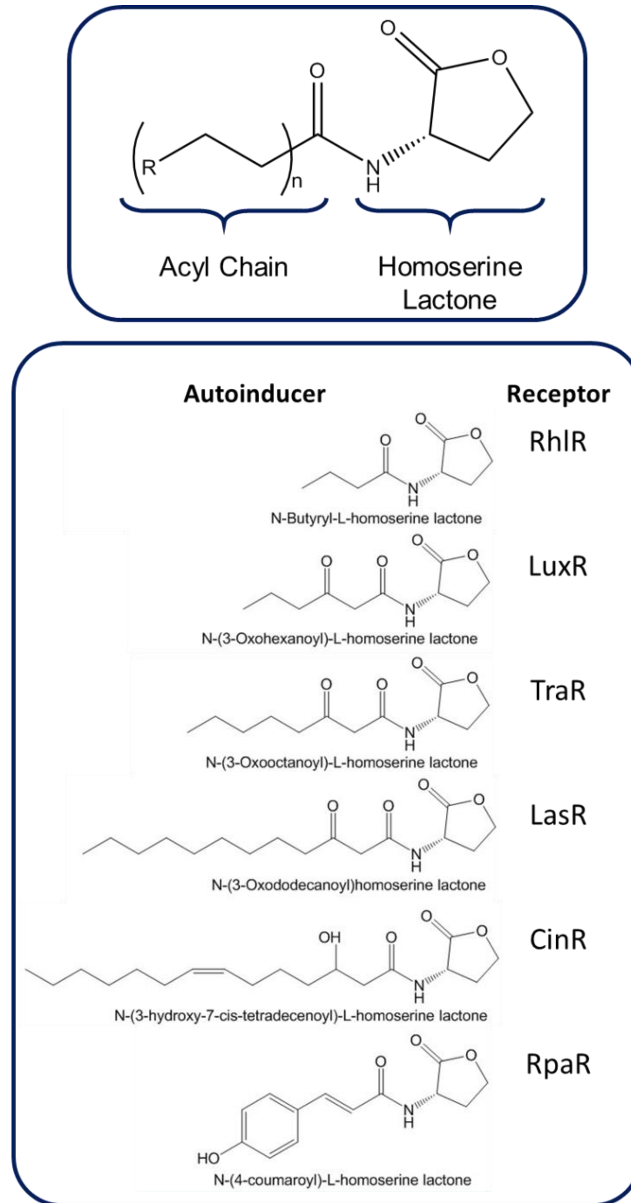


Fig 3. Quorum sensing signals containing characteristic homoserine lactone. General structure of acyl homoserine lactones, containing homoserine lactone and acyl fatty acid chain. Length and structure of acyl chain varies in different quorum sensing systems. LuxIR type quorum sensing signaling molecules (autoinducers) with varying structures, their cognate receptors are shown.

Rhodopseudomonas palustris contains LuxIR type pair, RpaI and RpaR. In *Rhodopseudomonas palustris,* LuxI type homoserine lactone synthase (RpaI) produces quorum-sensing signal pC-HSL, an aryl-homoserine lactone, from environmentally acquired p-coumarate (an aromatic lignin monomer) instead of endogenous fatty acid biosynthesis derivatives unlike typical other LuxI type synthases (Schaefer et al. 2008). This allows *Rhodopseudomonas palustris* to sense the level of plant biomass available for consumption and population density.

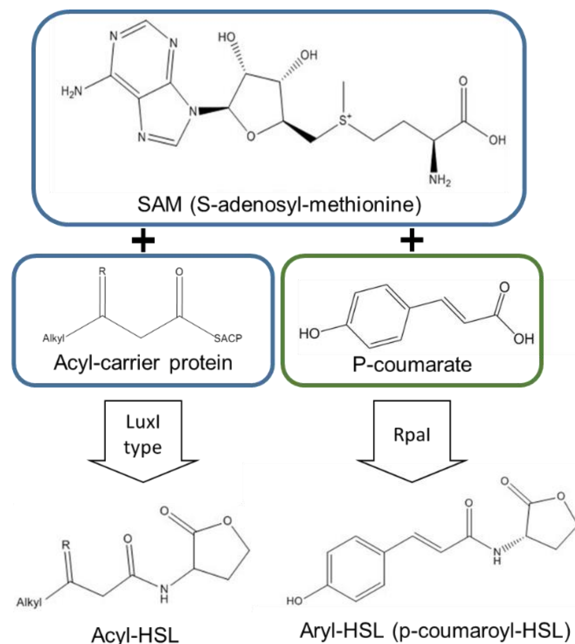


Fig 4. Illustration of typical acyl homoserine lactone synthesis in comparison to RpaI directed synthesis. RpaI uses a plant metabolite p-coumarate unlike typical LuxI type synthases.

HSL based quorum sensing machinery is seen in many species with HSL molecules that contain acyl chain modifications (for some quorum sensing systems see fig 3). This structural similarity between different HSL based quorum sensing systems can lead to activation of non-cognate systems (Hawver, Jung, and Ng 2016). There are two types of crosstalk that can occur between different quorum sensing systems. Due to the similarities of HSL molecule structures, binding of an HSL molecule to a non-cognate receptor is possible. It can cause activation of non-specific gene cascades. This type of crosstalk is called chemical crosstalk or signal crosstalk.

Similarly, a promoter can interact with a non-cognate receptor leading to activation of an unspecific gene cascade. This type of crosstalk is called genetic crosstalk or promoter crosstalk. (Kylilis et al. 2018). This is a problem for the use of quorum sensing architecture in synthetic circuits to connect different subpopulations. To reliably wire devices in a system, each input should result in the intended output instead of activating a nonspecific one. In this context, orthogonal refers to signaling architectures that do not crosstalk with each other. Having a repertoire of orthogonal signals available is valuable for scalability of multicellular engineering approaches. A recent study showed that there some possible combinations of QS systems that do not show significant crosstalk thus suitable for use in synthetic circuits (Kylilis et al. 2018; Scott and Hasty 2016).

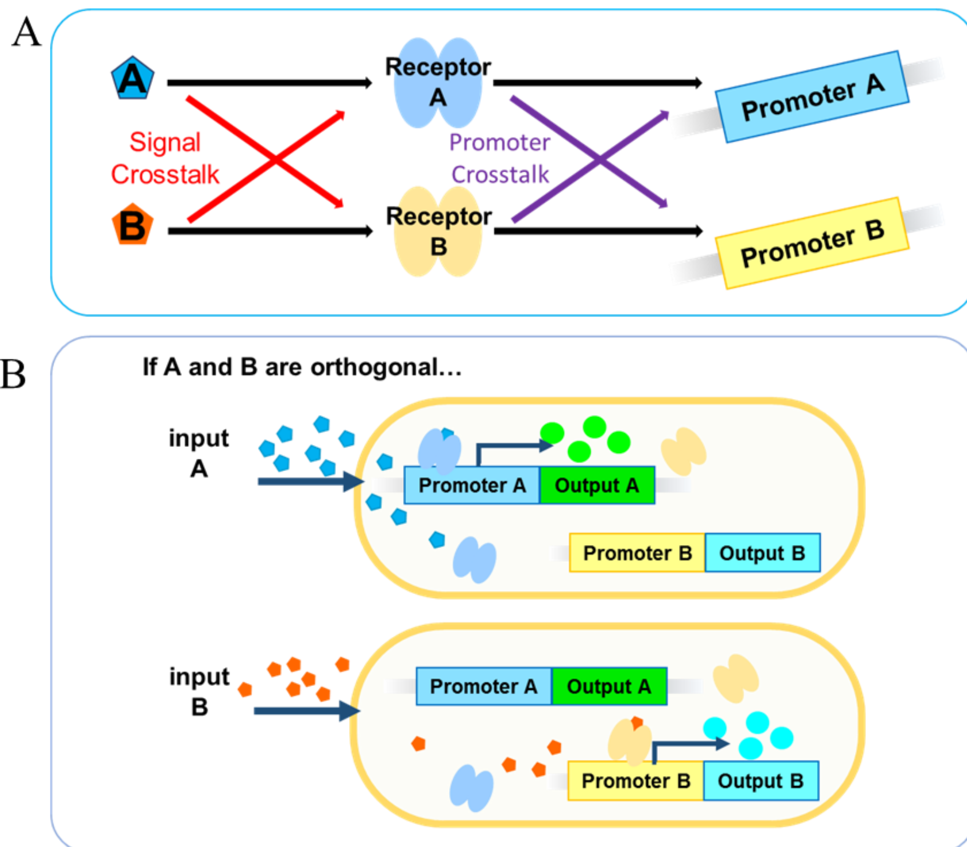


Fig 5. Crosstalk in quorum sensing systems and orthogonality is illustrated. **A** There are two types of crosstalk: signal crosstalk and promoter crosstalk. Signal crosstalk occurs when a quorum sensing autoinducer molecule binds to a non-cognate receptor that activates a non-cognate promoter (shown in the figure with red arrows). Promoter crosstalk is the activation of a promoter by a non-cognate receptor (shown with purple arrows in the figure). If two quorum sensing systems are orthogonal the auto inducer binds to its

cognate receptor and the autoinducer-receptor complex acts on the cognate promoter. **B** The scenario of two orthogonal quorum sensing system elements in a synthetic circuit is illustrated. In such circuit input A only results in the output A not output A and vice versa.

1.5. Current Limitations

AHL based quorum sensing has attracted the attention of synthetic biologists due to its simple architecture with three components: promoter, signaling molecule and receptor. The most basic use of quorum sensing in synthetic systems is its cell-to-cell coupling ability. Cell-to-cell coupling can synchronize the cells since diffusible signaling molecules reach to the entire population. One example to use of quorum sensing for this purpose is a genetic clock built by using LuxIR quorum sensing system along with a homoserine lactonase enzyme (Danino et al. 2010). In this study, quorum sensing signals not only facilitated the coordinated behavior of cells but also formed a negative feedback loop. With this negative feedback loop, the circuit exhibited synchronized oscillatory expression pattern. In synthetic circuits, the ability to turn off the system is beneficial for many applications, indeed. Homoserine lactone degradation enzymes are also called quorum quenching enzymes as they inhibit quorum sensing behavior in wide range of organisms from various bacteria to human epithelia. There are two groups of quorum quenching enzymes: HSL-lactonase and HSL-acylase that hydrolyzes lactone bond and peptide linkage of lactonase ring of the homoserine lactones respectively (Yi Hu Dong and Zhang 2005). The lactonase enzyme that degrades acyl homoserine lactones is coded by autoinducer inactivation gene (aiiA) (Y H Dong et al. 2000). Quorum quenching enzymes are suitable for use as an off-switch to quorum sensing based synthetic circuits.

Use of quorum sensing in synthetic biology is not only limited to synchronization of a homogenous cell population. Quorum sensing machinery can used to ensure communication and coordination between different subpopulations in microbial consortia. An oscillatory circuit built with a similar design principle with two specialized sub-populations. In this system, the genetic circuit is compartmentalized in sender and receiver cells (Y. Chen et al. 2015). Another example of a synthetic multicellular consortia with communicating subpopulations is a circuit built with NOR gates combined in four different cell types (Tamsir, Tabor, and Voigt 2011). Studies show that to build increasingly complex

devices, layering multiple specialized populations is possible (Y. Chen et al. 2015; Tamsir, Tabor, and Voigt 2011).

One of the major challenges limiting implementation of labor division concept is the difficulty of studying multiple cell populations in a controlled environment. In the previously mentioned synthetic multicellular systems, it is worth noting that the methods used to study the interactions of subpopulations have limited control over individual subpopulations. The interaction of four-way layered NOR gate was studied on agar plate where each population is spotted separately with equal distance (Tamsir, Tabor, and Voigt 2011). In two-way oscillatory sender-receiver system, a microfluidics device is used however subpopulations were grown in the same cell trap in mixed fashion. In both methods used there is limited control over the subpopulations. This might perhaps allow the over growth of subpopulations with better fitness in comparison to other populations. This kind of uncertainty can further complicate the dynamic of the microbiota. A recent publication by developed a method to study multicellular interactions in a controlled microfluidic setup where subpopulations are physically separated (Osmekhina et al. 2018). This shows that the current limitations presented can be tacked with promising microfluids solution and increasing resolution of printing technologies.

2. Aims

Our objective in the scope of this work is to construct a robust synthetic consortium consisting four different cell populations in order to explore the concept of labor division. Construction of this consortium allows us to study complex interaction patterns between subpopulations including negative and positive feedback loops. We aim to first individually characterize the performance of each subpopulation. Following the individual characterization, our final aim is to study the collective behavior of the consortium in a microfluidics setup that is being developed in house.

3. Materials and Methods

3.1. Construction of a Toolbox

The *E. coli* strain used for cloning and plasmid propagation was Top10 due to the high transformation efficiency and ability to replicate high copy number plasmids. For cloning, traditional restriction-ligation method was used with FastDigest restriction enzymes (Thermo Scientific) (type 2 restriction enzymes) and T4 DNA Ligase (New England BioLabs). *E.coli* strain, CY008, created by Bennett lab (Y. Chen et al. 2015) from Addgene containing ΔlacI ΔaraC ΔsdiA knockouts, was used as host for all experiments. In this study, two sets of plasmids were constructed; one containing the promoter-fluorescent protein pair(s) and another set containing receptor(s). Promoter-fluorescent protein pair was cloned to a pColE1 derivative backbone containing kanamycin. For the selection of pColE1 plasmid, kanamycin was used at 50µg/ml final concentration (from 50 mg/ml stock by 1:1000 dilution). pColE1 backbone contains a high copy number origin of replication, ColE1. Receptors were cloned to pACYC derivative backbone that contains ampicillin resistance gene and low copy number origin of replication (P15A). pACYC plasmid was selected with 100µg/ml final concentration of ampicillin (from 100 mg/ml stock by 1:1000 dilution).

The parts of Cin system, pCin and CinR are from *Rhizobium leguminosarum*. pRpa and RpaR are from *Rhodopseudomonas palustris*. A ribosomal binding site from Lux quorum sensing system was added to promoters. In this study, four different fluorescent proteins were used; eGFP (Danino et al. 2010), eCFP, mKO_2(Sakaue-sawano et al. 2008) and mCherry (Shaner et al. 2004). Cloned constructs are confirmed by colony PCR and sequencing. We used Eurofins Genomics TubeSeq service with premixed primers. (see the appendix for primer list)

3.2. Characterization

3.2.1. Transformation strategy

Plasmids containing respectively the promoter-fluorescent protein pair and its cognate receptor were transformed together into CY008. Kanamycin and ampicillin resistance

genes in the plasmid backbones were used as selective markers. Chemically competent CY008 cells were transformed with heat-shock method. Transformants were plated on Luria-Bertani (LB) agar plates and incubated overnight at 37 °C. From the LB-agar plates with transformed cells, single colonies were cultured in liquid LB media with both kanamycin and ampicillin overnight. From the overnight culture 25% glycerol containing stocks were prepared for storage at -80 °C.

3.2.2. Fluorescence Measurements with Plate-Reader

3.2.2.1. Sample preparation

In order to confirm that the constructs were functional, and to characterize the fluorescent protein expression levels, induction experiments were conducted with varying concentrations of externally added inducers. Synthetic inducer molecules, N-(-p-Coumaroyl)-L-homoserne lactone and N-(3-Hydroxytetradecanoyl)-DL-homoserine lactone were obtained from Sigma-Aldrich. The inducers were dissolved in 100% dimethylsulfoxside (DMSO). P-coumaric acid (Sigma-Aldrich) was also dissolved in DMSO. L-(+)-Arabinose (Sigma-Aldrich) was dissolved in deionized water and filter sterilized. The cells containing promoter-fluorescent protein and cognate receptor pair was cultured from previously prepared -80 °C glycerol stock. Cells were cultured overnight at 180 rpm at 37 °C. The next day, cells were diluted 1:1000 and re-cultured. The subculture was kept at 37 °C at 180 rpm shaking for 4 hours before the fluorescent measurements.

3.2.2.2. Fluorescence Intensity Measurements with Plate-Reader

Following the sample preparation, 200 µl of subculture was transferred to 96-well plates with corresponding inducer molecules (and precursors if needed). To test RpaI activity, the precursor p-coumaric acid was added externally. All experiments conducted with 3 experimental replicates. Then 96 well plate is scanned for following; endpoint absorbance for optic density (OD) at 600nm and fluorescence intensity (see the appendix) by Cytation3 by BioTek. Fluorescent intensity and OD was measured every 10 minutes for minimum 10 hours (for excitation and emission values see the appendix).

3.3. Co-cultures

For co-culture experiments, a similar protocol to previously described sample preparation for homogeneous cultures was followed; overnight pre-culture was inoculated to 2ml LB with necessary antibiotics and cultivated for 4 hours at 37 ^{o}C at 180 rpm. Two cell types that paired together were mixed 1:1 ratio in 96 well plate by addition of 100 µl from each culture. The inducer molecules were added to reach their final concentration in total volume of 200µl.

3.4. Flow Cytometry

3.4.1. Sample preparation

An overnight pre-culture was inoculated with CY008 cells containing both kanamycin and ampicillin backbones from -80 ^{o}C glycerol stock. A subculture was set by adding 2µl of pre-culture to 2ml fresh LB medium with ampicillin and kanamycin (1 to 1000 dilution). The sub-culture was incubated for 4 hours at 37°C at 180 rpm. 200µl of the incubated subculture was transferred to 96-well plate and necessary inducers are added. The 96-well plate was cultured for 4 hours in Cytation3 plate reader at 37°C with shaking. After 4 hours, the induced cultures were diluted in 1 X filter sterilized PBS solution with 1:10 ratio and stored overnight at +4 ^{o}C. Before the measurement on the following day cells in PBS were diluted again in 1X filter sterilized PBS with 1:2 ratio reaching to 1:20 from the original culture.

3.4.2. Flow Cytometry Measurements

For flow cytometry, BD FACSAria III (BD Biosciences) was used. For each sample, a minimum of 10000 events recorded. For the measurement, the parameters set at FSC 200 voltages, SSC 286 voltages, AmCyan-A 470 (for GFP) and PE-Texas Red-A 600 (for mKO2). Flow rate during data acquisition was 4µl/min. Data was analyzed and gated using the BD FACSDiva 8.0.1 software.

3.5. Microfluidics Chip Fabrication

To fabricate microfluidic chips to be used in combination with fluorescent microscopy, a 1 to 10 ratio of curing agent to silicone elastomer was used (SYLGARD 184). The mixture of silicone and curing agent was degassed with a vacuum pump to remove bubbles formed during mixing. The degasified mixture was poured to an SU-8 mold and baked at 80 ^{o}C for 5 hours. The mold used in this study were obtained from collaboration with Materials for Electronics research group of Prof. Sami Franssila. The chips were cut out carefully out with a scalpel. The inlet and outlet ports were punched with a 1,5 µm and 3 µm diameter respectively. The chips were treated by submerging in n-pentane for 2 hours to remove any unbound silicone monomers. After 2 hours, n-pentane was removed, and the chips were submerged in acetone for 1 hour. After acetone treatment, for the final rinse the chips were submerged in distilled water for 1 hour to remove traces of solvent absorbed into the chips. After the wash steps, chips were dried with a blow-dryer individually from both sides and left in a sterile petri dish overnight to dry. The glass coverslips were cleaned with 70% ethanol and Kimwipe delicate task wipers (from Kimtech) and dried with blow-dryer. It rinsed with water and dried with blow-dryer again. This step was repeated until there were no dust particles visible. Dried chips were bonded to the coverslip with plasma surface treatment machine (Zepto from Diener Electronic). Bonded chips were 3-D printed with IP photoresist IPL 780 from Nanoscribe by using two-photon polymerization system (Nanoscribe) at the University of Jyväskylä Nanoscience Center .

4. Results

4.1. Construction of a Toolbox

In this study, we aimed to build a multicellular synthetic consortium that communicates using quorum sensing machinery. For this purpose, we constructed two sets of parts: promoter-fluorescent protein pairs and receptors. Fluorescent proteins tagged with degradation sequence LAA are used as reporters. Cin and Rpa systems are used since they show minimal crosstalk with each other (based on literature and in house unpublished data) (Kylilis et al. 2018). We used native Rpa and Cin promoters (pCin and pRpa) fused with

Lux ribosomal-binding site. The fused promoters are expressed in combination with their native receptors RpaR and CinR in bacteria. Downstream to the promoters, we cloned fluorescent proteins. In this system, quorum sensing signaling molecule and its cognate receptor forms a complex that binds to promoter. This leads to expression of fluorescent reporter. This modified quorum sensing architecture performs a basic function: sensing an incoming signal and indicating that the signal is received. We also constructed another basic function, signal production. For this purpose, we incorporated quorum sensing signal molecule synthase genes (CinI and RpaI). With these two functions one cell type can receive a signal and produce a signal that can induce another cell type. (Fig 7) For example, a cell type that contains Cin promoter fused with GFP reporter and CinR receptor will express GFP in the presence of Cin signal. In this case, the cell receives its specific signal and reports it by expression of GFP. Addition of quorum sensing signal synthase this circuit integrates the signal production function. If we put RpaI gene under the control of a second copy of pCin promoter, Cin-CinR signal will not only induce expression of GFP but also production of a secondary QS signal, Rpa. Consecutively, produced Rpa signal can be received by another cell type.

There are many fluorescent proteins with varying properties used for imaging. When using multiple fluorescent proteins together, overlap of excitation and emission curves can make distinguishing between two fluorescent proteins difficult. As we aimed to engineer four cell types with different fluorescent reporters imaging fluorescent proteins with minimum interference was essential. Therefore, we selected eGFP, eCFP, mKO2 and mCherry. Using the peak excitation and emission values for each fluorescent protein, we noted the percentage of excitation and emission overlapping from other fluorescent proteins. For this, we used Spectra Viewer tool by Chroma Technology. We used mKO fluorescent spectrum since mKO2 was not available and mKO2 fluorescent protein is the a fast-folding variant of mKO fluorescent protein. For each fluorescent protein, we calculated the percentage of unspecific signal that bleeds to the channel used (figure 1). Based on our calculations, 0.33% mKO bleeds to eGFP channel and 2.32% eCFP bleeds to eGFP channel.

eCFP		Ex %	Em %	Detection %
	eGFP	29.65	0	0
	mKO	1.34	0	0
	mCherry	0	0	0

eGFP		Ex %	Em %	Detection %
	eCFP	3.68	63.30	2.32
	mKO	18.74	0	0
	mCherry	8.08	0	0

mKO		Ex %	Em %	Detection %
	eCFP	0	12.60	0
	eGFP	2.09	16.10	0.33
	mCherry	56.95	0	0

mCherry		Ex %	Em %	Detection %
	eCFP	0	0	0
	eGFP	0	2.10	0
	mKO	0	13.48	0

Fig 6. Percentage of bleeding to unspecific channels is calculated for each fluorescent protein.

Table 1. The list of the promoter-fluorescent protein pairs constructed.

	Promoter	Flourescent Protein	Promoter	Synthase
1	pCin	GFP	-	-
2	pCin	CFP	-	-
3	pCin	mKO2	-	-
4	pCin	mCherry	-	-
5	pCin	GFP	pCin	CinI
6	pCin	CFP	pCin	CinI
7	pCin	mKO2	pCin	CinI
8	pRpa	GFP	-	-
9	pRpa	CFP	-	-
10	pRpa	mKO2	-	-
11	pRpa	mCherry	-	-
12	pRpa	GFP	pRpa	CinI
13	pRpa	CFP	pCin	CinI
14	pRpa	CFP	pCin	RpaI
15	pRpa	CFP	pRpa	CinI

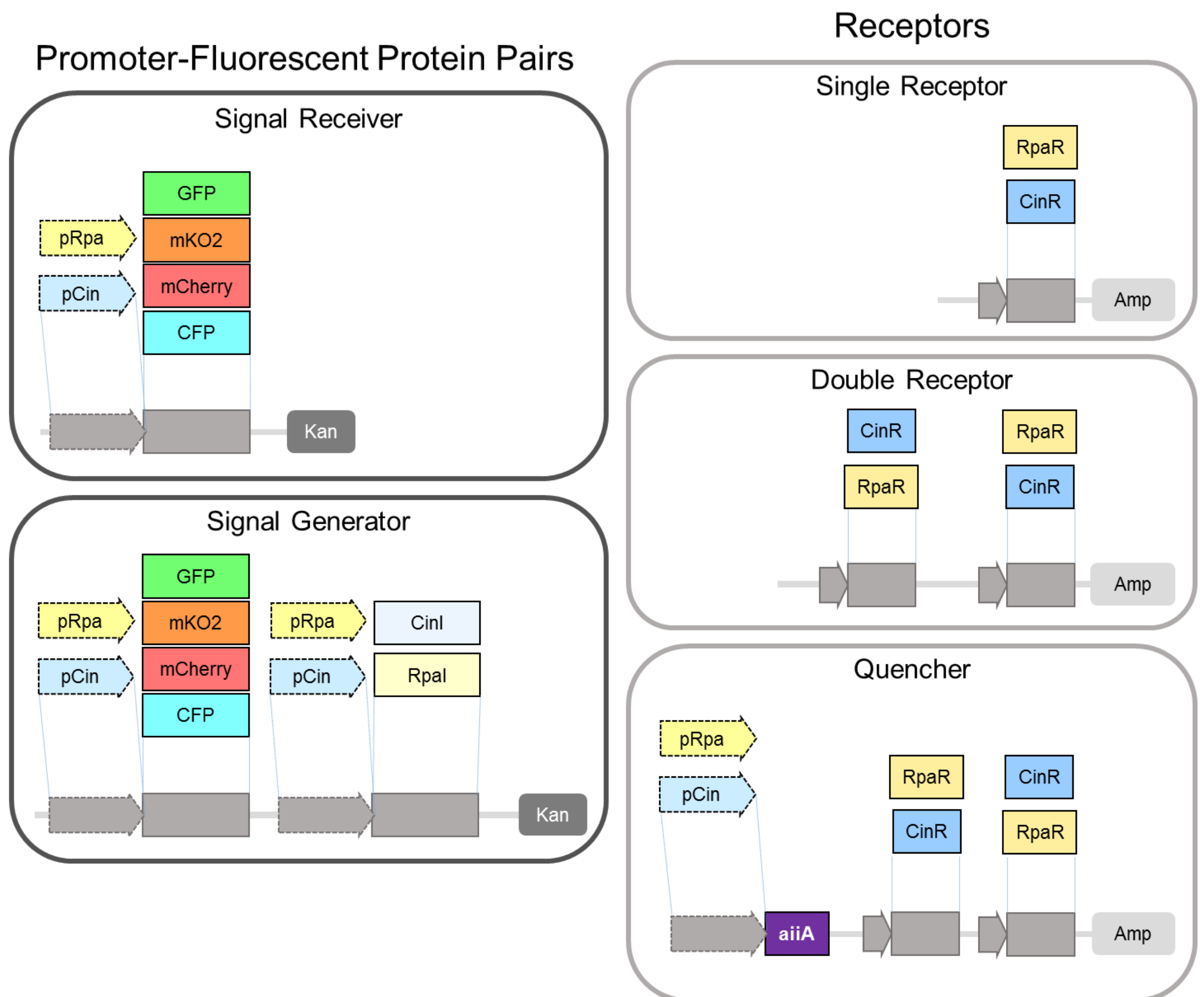


Fig 7. Design library is illustrated. We designed two sets of plasmids: for quorum sensing promoter-fluorescent protein pair(s) and for quorum sensing receptors. Promoter-fluorescent protein pairs were transformed together with receptor plasmids that contains the cognate receptor of the promoter. Signal receiver part receives a quorum signal and produces a fluorescent protein indicating that the signal is received. Signal receiver produces a fluorescent protein when signal obtained. Signal generator part, in addition to fluorescent protein synthesis, directs homoserine lactone synthesis resulting in quorum sensing signal generation. Receptor set contains single receptors, double receptors and a circuit termed quencher that contains double receptor in addition to inducible homoserine lactonase gene *aiiA*. Receptors (denoted as *CinR* and *RpaR*) were placed under constitutive promoters.

For this work 15 promoter-fluorescent protein pairs were cloned, and sequence confirmed. (Table 1) Eight of them are signal receiver parts and seven are signal generators.

We also used two plasmids containing arabinose inducible synthase genes to test the function of synthase genes. Both single receptors were available in house. We constructed double receptor and quencher.

4.2. Characterization of the Parts

4.2.1. Signal receiver

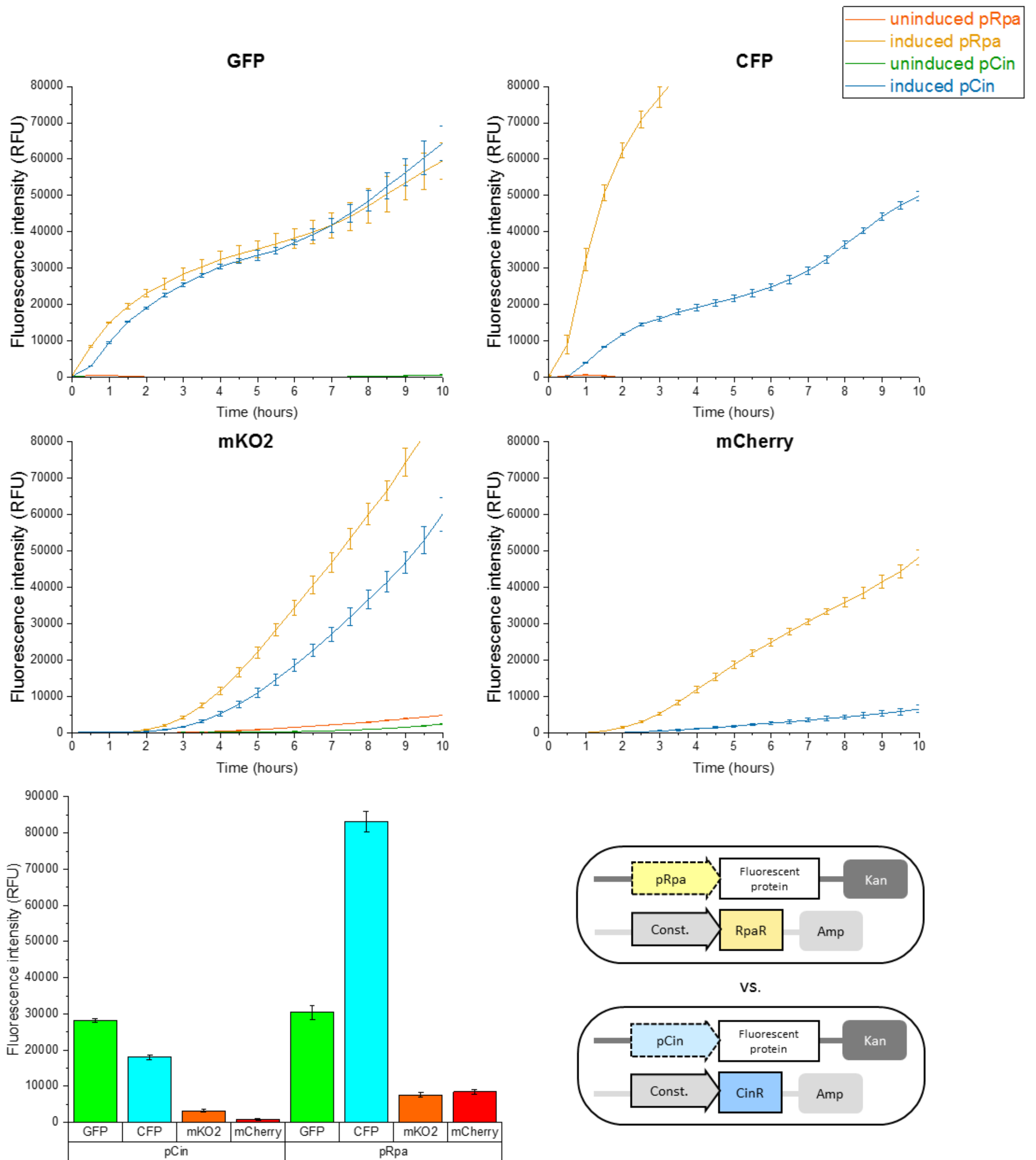


Fig 8. Comparison of fluorescent protein expression in E. coli strain CY008 under Rpa and Cin promoters over time. **A, B, C, D** Normalized fluorescent intensity (RFU) levels compared from Cin promoter and Rpa promoter. circuits expressing GFP, CFP, mKO2 and mCherry fluorescent proteins, repectively. **e** Normalized fluorescence intensity of GFP, CFP, mKO2 and mCherry 3.5 hours after induction of pCin and pRpa promoters. **F** Illustration of circuits containing pRpa and pCin inducible promoters upstream to fluorescent proteins compared. RpaR and CinR denotes cognate receptor proteins acting as transcription factors when bound to their cognate HSL. Receptor proteins, RpaR and CinR are under a constitutive promoter demonstrated as grey arrow.

Eight signal receiver parts were constructed with four fluorescent proteins containing pCin or pRpa promoters. To test their functionality we induced pCin and pRpa promoters with N-(3-Hydroxytetradecanoyl)-DL-homoserine lactone (Cin) and N-(-p-Coumaroyl-L-homoserine lactone) (Rpa). We cultivated CY008 cells containing pCin or pRpa and cognate receptors were induced with corresponding quorum sensing molecule (autoinducer) at the same final concentration of 10^{-6}M (1µM). (Fig 8F) Recorded relative fluorescence intensity was normalized with OD 600 and plotted over time. We compared each fluorescent protein under the control of *pRpa* and *pCin* promoters. (Fig 8) Fluorescence intensity of GFP protein follows the same trend under *pCin* and *pRpa* promoters throughout the duration of the experiment. (Fig 8B) However, other fluorescent proteins, CFP, mKO2 and mCherry, under *pRpa* promoter show higher fluorescent levels than fluorescent proteins downstream to *pCin* promoter. (Fig 8B, C, D) Fluorescence intensity of cyan fluorescent protein (CFP) induced with Rpa under pRpa promoter exceeded 80000 RFU after 3 hours, exceeding the measurement limit, whereas the fluorescence intensity of CFP expressing population with induction of pCin promoter only reached to 50000 in 10 hours. (Fig 8B) Similarly for mKO2 and mCherry, fluorescence intensity levels showed a significant difference under pCin and pRpa promoters. (Fig 8C, D) Notably after 5 hours of cultivation, we observed increasing levels of fluorescence intensity from uninduced population of both pCin-mKO2 and pRpa-mKO2**.** Higher levels of fluorescent intensities measured under the control of pRpa promoter. (Fig 8C) We compared normalized relative fluorescent intensities (rfu) of GFP, CFP, mKO_2 and mCherry at the 3.5 hours' time point under pCin and pRpa promoters. (Fig 8E) 3.5 hour after induction, under pCin promoter, GFP had the highest intensity above 25000 rfu and CFP was the second highest after GFP

at ~20000 rfu. The levels of mKO2 and mCherry under pCin were below 10000 rfu. Under pRpa promoter levels of mKO2 and mCherry were higher than pCin-mKO2 and pCin-mCherry. Under control of the pRpa promoter, CFP reached the highest fluorescence intensity over 80000 rfu, which was approximately 10-fold more than mKO2.

4.2.2. OFF-switch

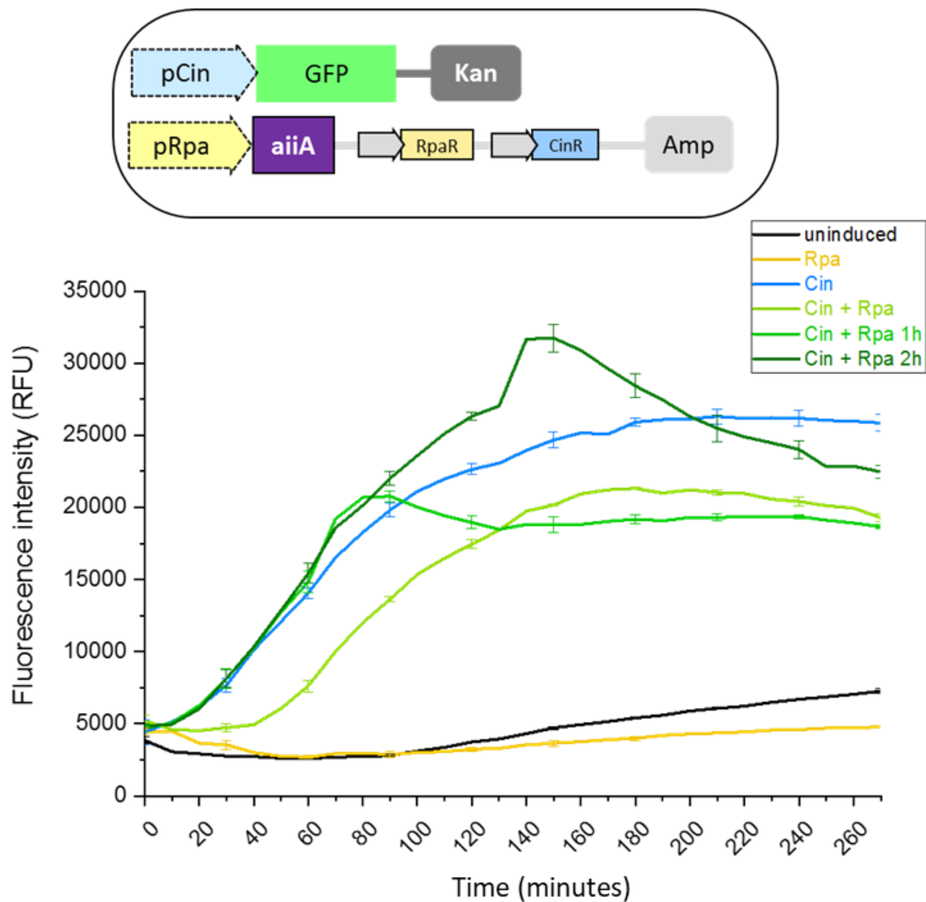


Fig. 9. Effect of double receptor with inducible homoserine lactonase. Illustration of the circuit that forms negative feedback loop by degradation of initial QS signal via Lactonase enzyme, denoted as aiiA in the purple box under inducible pRpa promoter. Fluorescent intensity of the circuit illustrated in was plotted over time. We induced populations with Rpa and Cin initially (0 hour), and additionally with Rpa after 1 and 2 hours.

We aimed to include a quorum quenching enzyme to our system since this type of turn off signal is vital for oscillatory behavior. The cell type shown in figure 6 contains signal receiver and signal quencher parts that are activated by separate quorum sensing signals,

Cin and Rpa consecutively. AiiA gene was combined with CinR and RpaR genes in the receptor backbone and paired with pCin-GFP signal receiver. (Fig 9) This cell type enables us to control both the signal receiver part and the signal quencher protein with external addition of signaling molecules individually. Fluorescence intensity level of eGFP was plotted (Fig 9). In the plot, there was an increase in fluorescence intensity level of uninduced population over time reaching to 10000 rfu after 10 hours. Whereas the intensity levels remained lower when aiiA was induced with Rpa. In Cin induced population, GFP levels reach the peak after 3.5 hours, around 25000 rfu and start decreasing. When the population was induced with both Rpa and Cin initially, a similar trend was observed, peaking after 3 hours at 20000 rfu. We also induced aiiA at 60 and 130 minutes (Cin+ 1h Rpa and Cin+ 2h Rpa, respectively) from both of these populations upon induction of the lactonase enzyme, aiiA, the GFP levels reaches to their peaks within the following 30 minutes and start decreasing. Both induced at 60- and 130-minutes drop to the 10000 rfu levels after 7 and 9.5 hours, respectively.

4.2.3. Distinguishing Signals

Using parts of different quorum sensing systems in combination in a cell type can potentially cause unreliable behavior of synthetic devices. One obvious reason behind this is possible crosstalk between parts, if the quorum sensing systems are not orthogonal. Another reason behind this could the unexpected effect of upstream or downstream sequences on each other. This is referred as context dependency. Also, addition of a constitutively expressed secondary receptor and an inducible gene can possibly cause metabolic burden on the expression host. Thus, we tested single receptor, double receptor and quencher parts. (Fig 10)

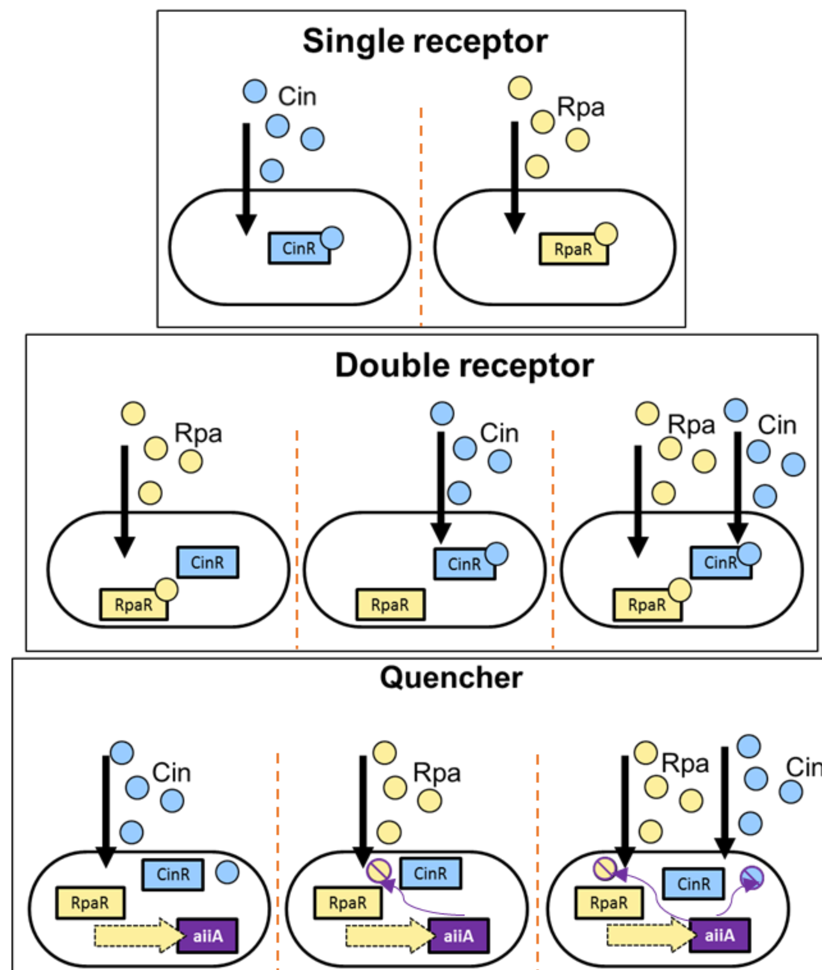


Fig 10. Illustration of different quorum sensing receptor organizations. Single receptors CinR and RpaR bind only with their cognate quorum sensing signals shown in blue and yellow respectively. When double receptors are included in a circuit, both receptor proteins are present in the cell. In this case, each receptor binds with their cognate receptor only in all scenarios. The quencher part represented in the illustration, contains quorum quenching enzyme (aiiA) under the control of Rpa promoter (yellow arrow). This means that aiiA is only expressed when Rpa signal is present thus in the presence of Cin, it does not degrade quorum sensing signal Cin. However, when Rpa is available in the cell, aiiA is expressed and degrades its own inducer molecule, Rpa. This results in reduced expression of aiiA, leading to negative self-regulation of aiiA. If both Cin and Rpa are present, aiiA degrades both quorum sensing signals.

In order to compare the performance of single receptor, double receptor and quencher in a circuit, we used a signal receiver part containing pCin-GFP. We only induced pCin promoter leading to GFP expression (Fig 11). GFP intensity from all three populations followed the same trend in first 3 hours and reached to approximately 25000 rfu. After 3.5 hours fluorescent intensity from population with single receptor increased to over

60000 rfu whereas population with double receptor reached 30000 rfu. After 3.5 hours' time point the fluorescent intensity of the population containing the double receptor with aiiA gene showed a decrease. Uninduced population containing aiiA showed higher levels of fluorescent intensity compared to other populations.

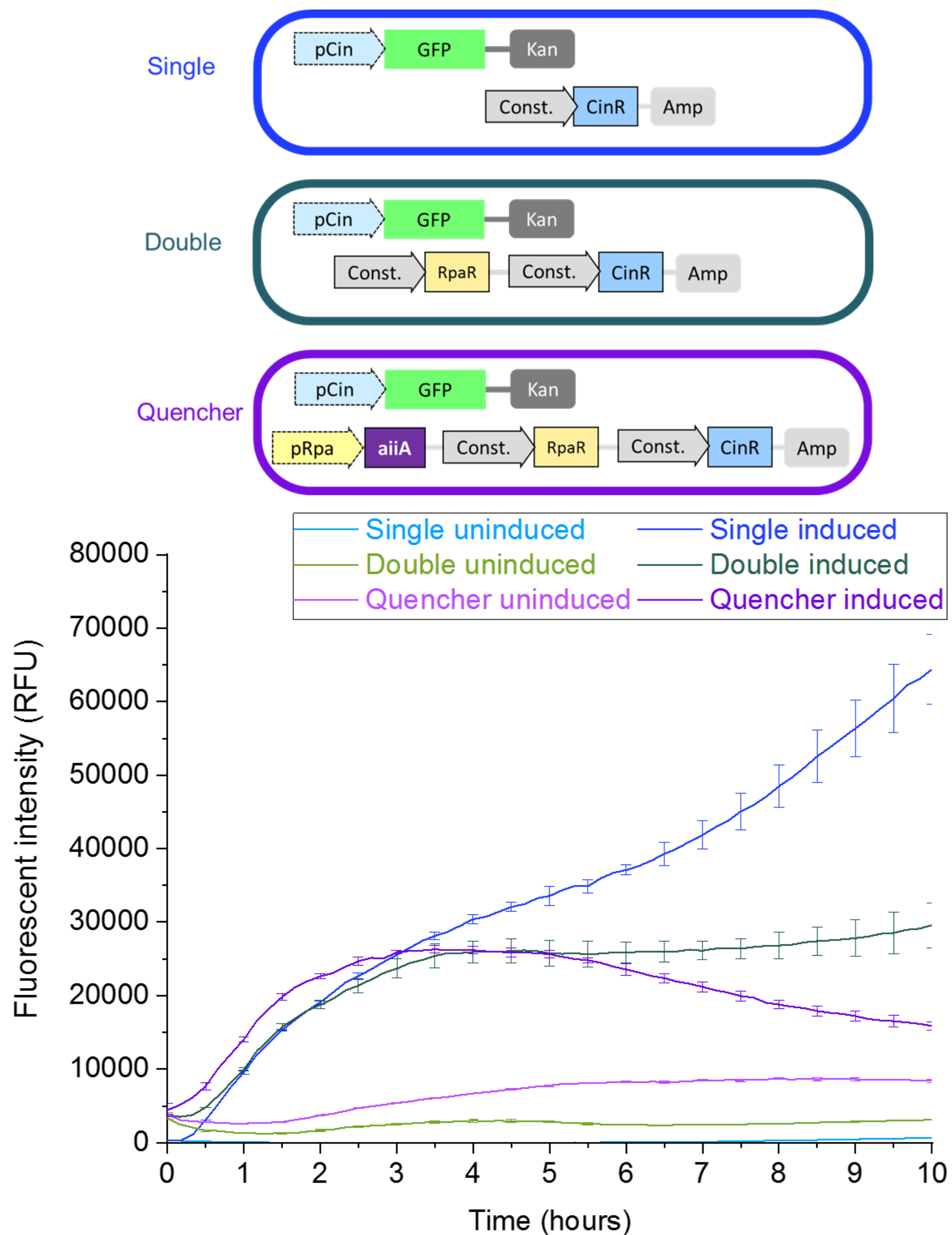


Fig 11. Comparison single receptor, double receptor and quencher. GFP fluorescence intensity plotted over time demonstrates the effect of double receptor and inducible lactonase enzyme on expression levels. The illustration shows the cell types compared: single (blue), double (green) and quencher (purple).

4.2.4. Signal Generation

4.2.4.1. CinI

In order to test the synthase gene *CinI*, we placed it under the control of arabinose inducible promoter. We used pCin-GFP pair to observe the levels of Cin signal produced. In this system, upon induction with arabinose CinI gene responsible from synthesis of N-(3-Hydroxy-7-cis-tetradecenoyl) homoserine lactone (referred as Cin) is activated. In the presence of Cin, it binds to CinR. This forms a complex and acts as a transcription factor for pCin promoter. When pCin promoter is activated, green fluorescent protein is expressed (Fig 12). We induced the cells with increasing concentrations (from 0,001mM to 10Mm) of Arabinose. The culture with 0,001mM final arabinose concentration (the lowest concentration used in this experiment) showed the highest fluorescent intensity at 70000 rfu, 14 hours after induction. Second highest fluorescent intensity was at 60000 rfu after 14 hours from the culture with 0,01mM arabinose concentration. At 0.1mM final concentration of arabinose fluorescent intensity reached to 10000 rfu after 15 hours. Uninduced population showed no significant fluorescent intensity as well as populations supplied with 1mM and 10mM of arabinose.

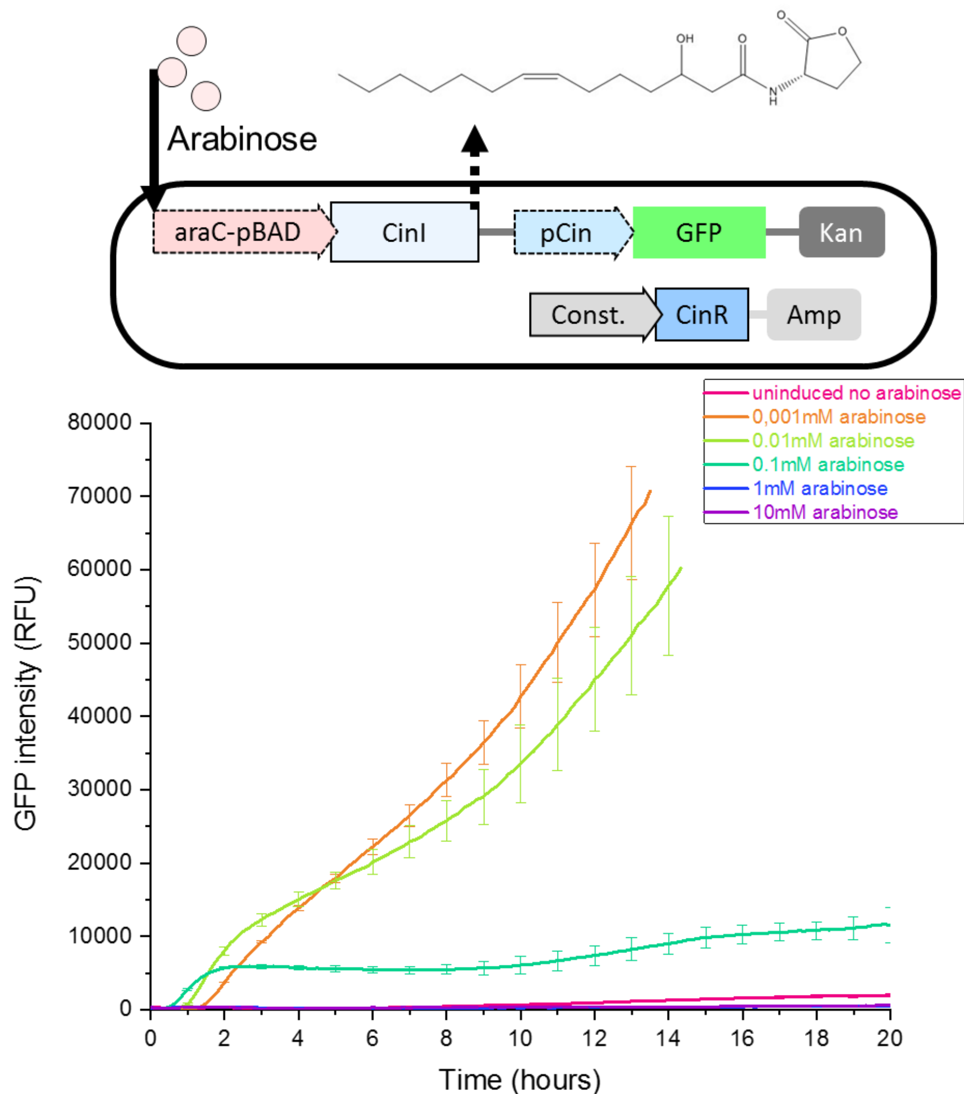


Fig 12. The illustrated cell type is induced with varying concentrations of arabinose. Normalized fluorescent intensities are plotted over time.

4.2.4.2. RpaI

We tested the activity of Rpa synthase (RpaI) under arabinose inducible promoter araC-pBAD. We coupled arabinose inducible RpaI with pRpa-GFP signal receiver part to observe the N-(-p-Coumaroyl)-L-homoserne lactone produced (Fig 13). In this circuit expression of RpaI begins when araC-pBAD promoter is induced with arabinose. This leads to expression of RpaI. RpaI facilitates N-(-p-Coumaroyl)-L-homoserne lactone synthesis. This induces the expression of GFP by activating pRpa promoter when bound to RpaR

receptor. We supplied the cultures only with arabinose at varying concentrations and measured fluorescent intensity of GFP. (Fig 13) At 10 mM final arabinose concentration, the highest fluorescent intensity reached to 20000 rfu 18 hour after induction. After 18 hours, culture induced with 1 mM final arabinose concentration reached the fluorescent intensity of 15000 rfu. Measured fluorescent intensities from populations with 0.01 mM and 0.001mM arabinose concentrations were at the same levels as the uninduced culture. This experiment is repeated twice to eliminate possible experimental error however both experiments showed similar results.

Fig 13. The illustrated cell type is induced with varying concentrations of arabinose. Normalized fluorescent intensities are plotted over time.

However, the levels of GFP was still much lover in comparison with external addition of Rpa signal. (Fig 8, Fig13) It was previously shown that RpaI uses a precursor molecule, p-coumarate to synthesize the N-(-p-Coumaroyl)-L-homoserne lactone (Amy L. Schaefer et al. 2008). Therefore, we tested the same circuit with externally added the precursor (p-coumaric acid) with 10mM arabinose induction. (Fig 14) The cell culture supplied with 10mM arabinose and 1mM p-coumaric acid had the highest fluorescent intensity at 70000 rfu after 15 hours. The highest p-coumaric acid concentration at 10mM not only led to growth defects but also to inhibited fluorescence protein expression. Addition of 5mM p-coumaric acid led low levels of fluorescent intensity up to 16 hours and started decreasing further after 16 hours as the cell density of the population decreased.

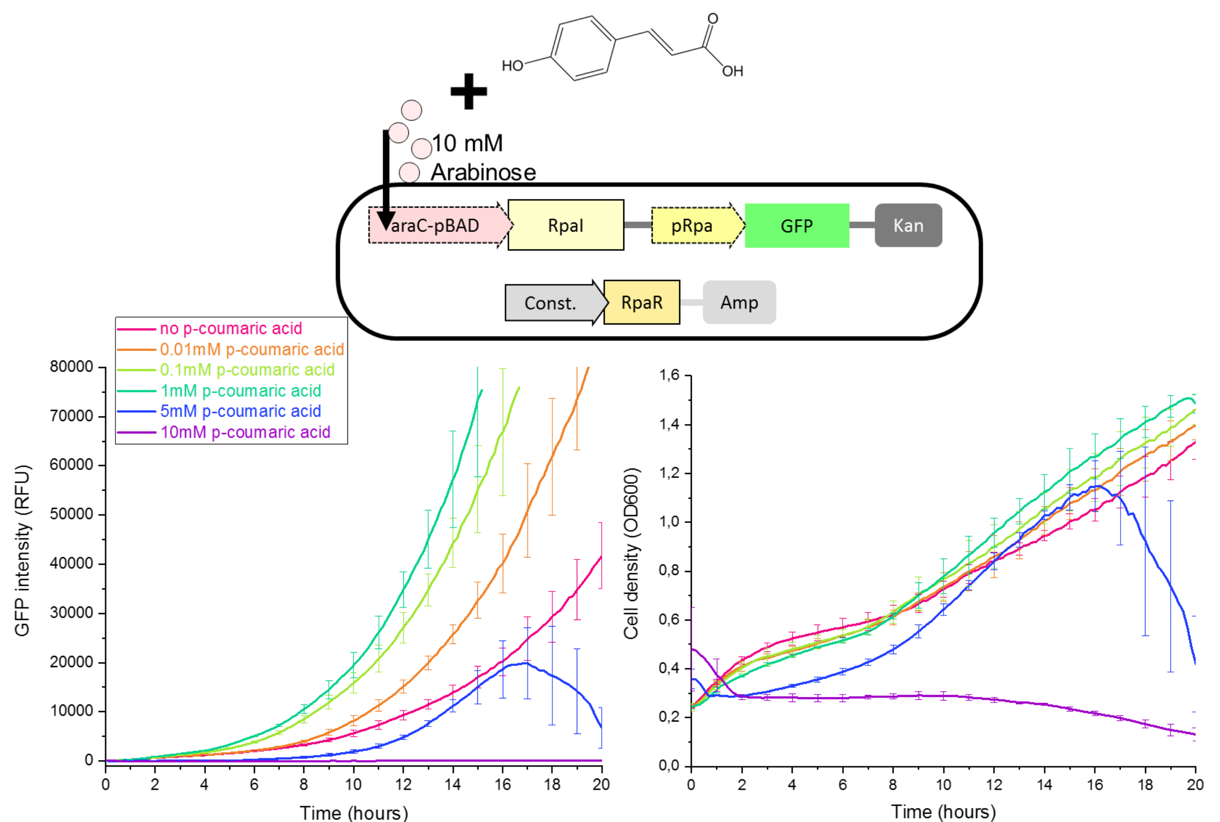


Fig 14: The illustrated cell type is supplied with varying concentration of p-coumaric acid and 10mM arabinose. Fluorescent intensities and cell densities are plotted over time.

4.3. Signal Transduction

We tested the signal transduction from one cell to another by performing co-culture experiments. In this setup, the paired cell types were sender and receiver. The sender contains signal receiver and generator parts. The receiver contains only signal receiver. First, we cultured the selected cells individually and tested their individual functions. The signal generator containing arabinose inducible RpaI and pCin-mKO$_2$ was induced with 10mM arabinose and 1μM (10^{-6} M) Cin signaling molecule. (Fig 15) Uninduced and 10mM arabinose added cultures did not show any significant fluorescent intensity until 8 hours. The fluorescent intensity of the induced sender reached to 60000 rfu after 12 hours. Fluorescent intensity of the receiver cell induced with 1μM (10^{-6} M) Rpa signaling molecule reached to 80000 rfu at 3 hours.

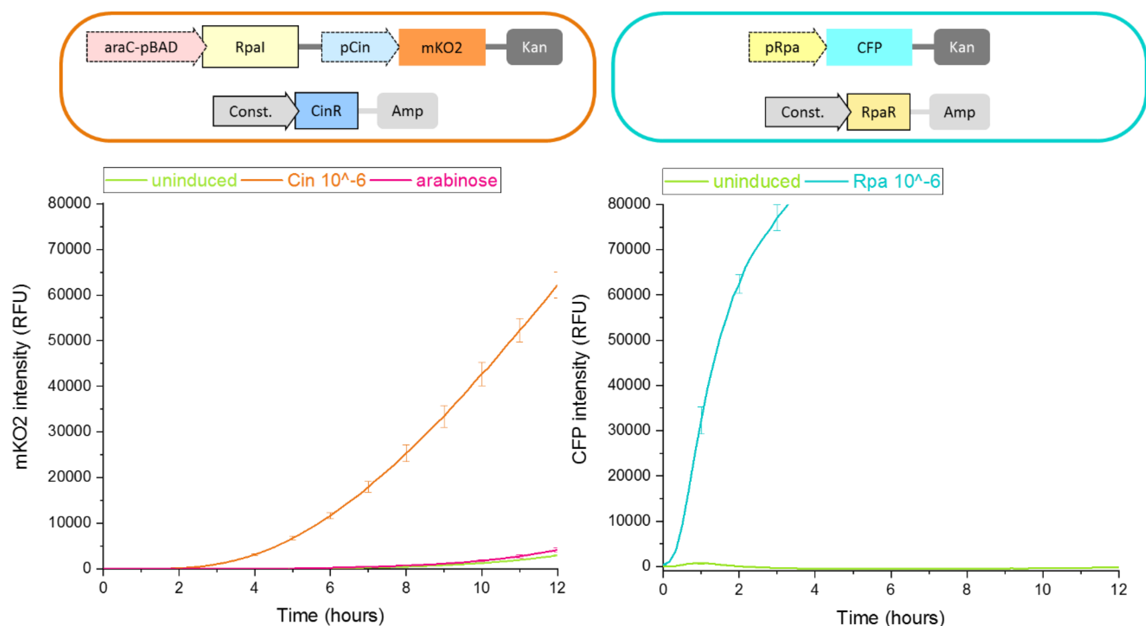


Fig 15. A sender and a receiver cell type were cultured separately. The sender and receiver cell types are illustrated. Normalized fluorescent intensities of sender (mKO2) and receiver (CFP) are plotted over time.

Following the individual characterization, sender and receiver cell types are cultured together. In the co-culture, sender and receiver subpopulations were expected to communicate via Rpa signal produced by the sender cells. (Fig 16) When the culture was induced with p-coumaric (1mM) acid and arabinose (10mM) fluorescent intensity of CFP reached to 70000 rfu in 12 hours.

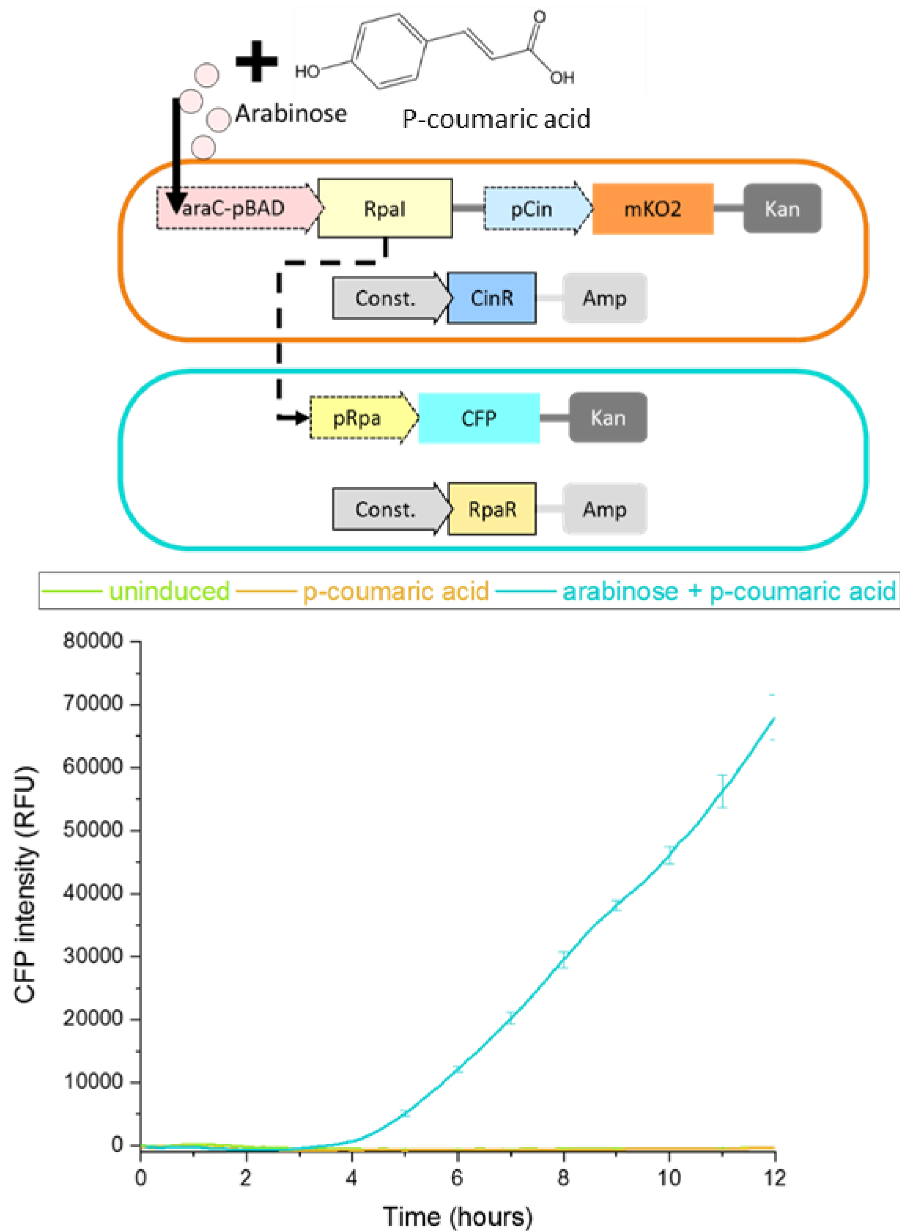


Fig 16. Signal transduction from one cell type to another was tested with co-culture of a sender and receiver pair. The cell illustrated with orange is signal sender. The sender synthesizes Rpa signal (dashed line). The cell type illustrated with blue is the receiver. The CFP expression by receiver cells, represents the signal transduction from sender to receiver. Fluorescent intensity of CFP from the co culture is plotted over time.

Another co-culture experiment is set up with a second sender and receiver pair that interacts via Cin signal molecule. Again first, we tested the subpopulations separately.

(Fig 17) The sender containing pRpa-GFP and pRpa-CinI parts was induced with 1µM of Rpa signaling molecule. The fluorescent levels of induced sender reached to 30000 rfu in 6 hours. (Fig 17) The receiver containing pCin-mKO2 part was induced with 1µM of Cin reached to 60000 rfu in 4 hours and exceeded 80000 rfu in less than 5 hours. Uninduced population of receiver showed mKO2 expression that reached to 35000 rfu in 12 hours.

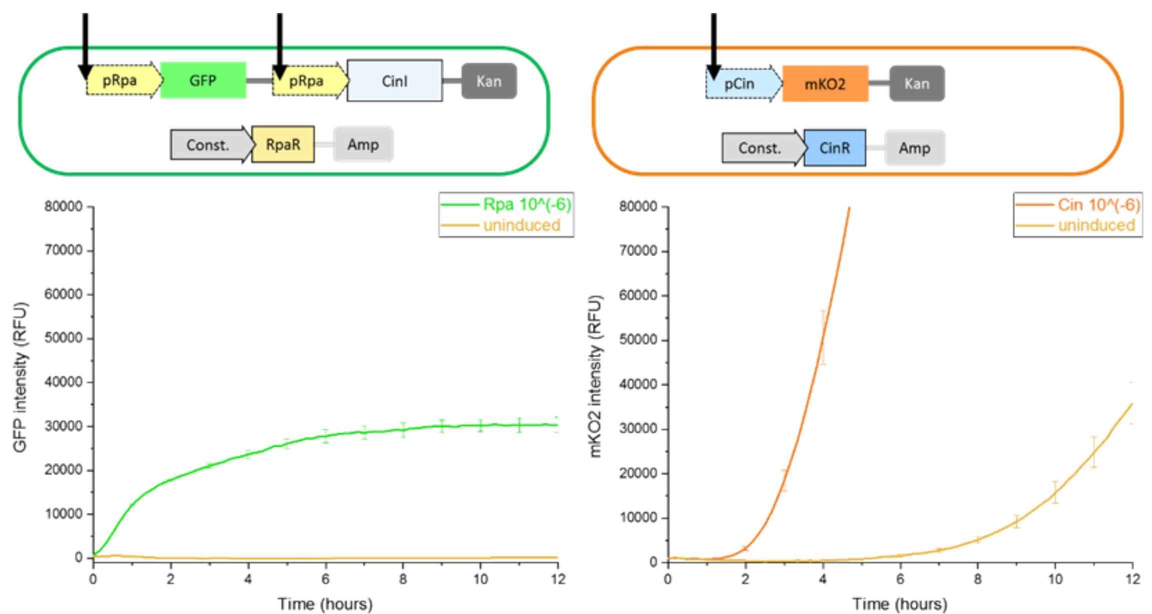


Fig 17. Individual induction experiment of subpopulations of a co-culture that communicates via Cin. **a** signal generator cell type (illustrated) that contains pRpa-GFP and pRpa-CinI pair. The signal generator was induced with Rpa and the fluorescent intensity from GFP is plotted over time. **b** The signal receptor that contains pCin-mKO_2 was induced with Cin. Fluorescent intensity of mKO_2 is plotted over time.

Following the individual characterization of two cell types were co-cultured together. (Fig18) The co-culture was induced with Rpa. Upon Rpa induction the sender expressed GFP and CinI gene that synthesize Cin signal. Cin is picked up by receiver cells resulting in mKO2 synthesis. From the co-culture, we measured both GFP and mKO2 fluorescent intensities for induced and uninduced samples. GFP fluorescent intensity in co-culture was approximately 3-fold less than homogeneous culture of signal generator at 10000 rfu. When the co-culture was induced with 1µM Rpa, mKO_2 fluorescent intensity of the signal receiver reached to 45000 rfu in 4 hours. The uninduced co-culture reached to 35000 rfu in 4 hours.

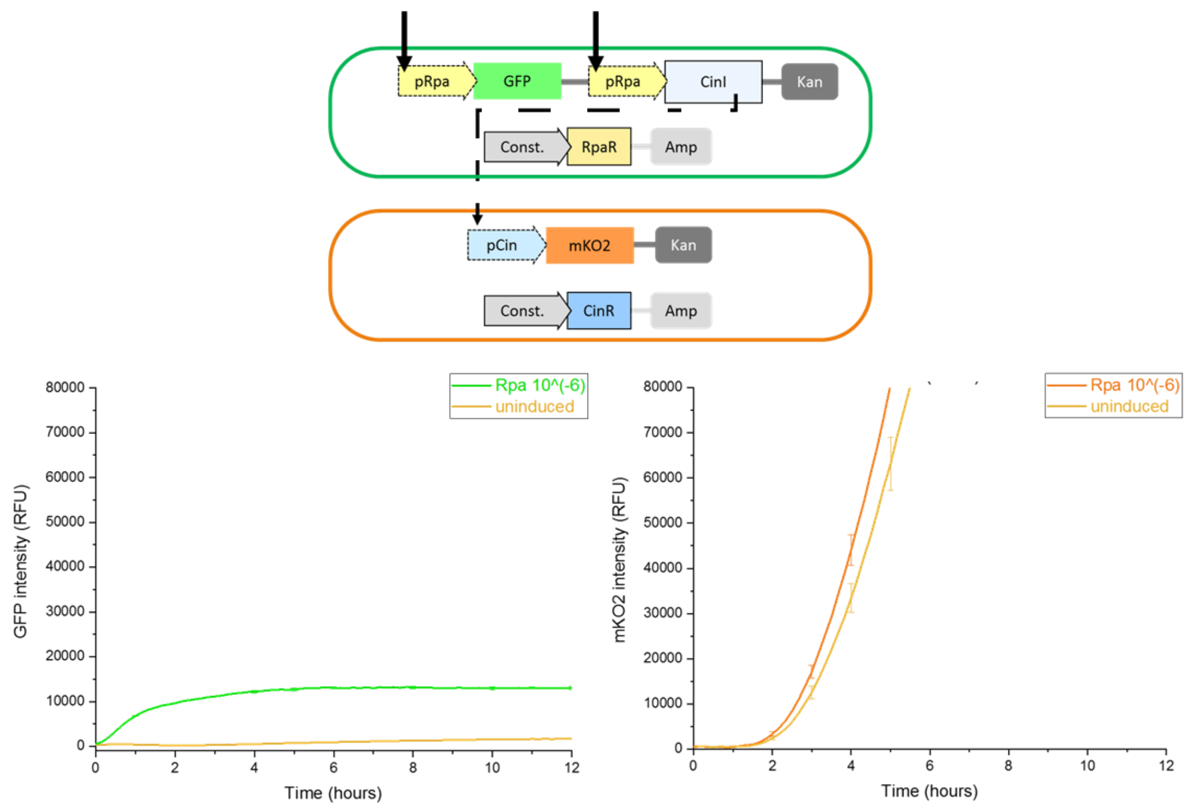
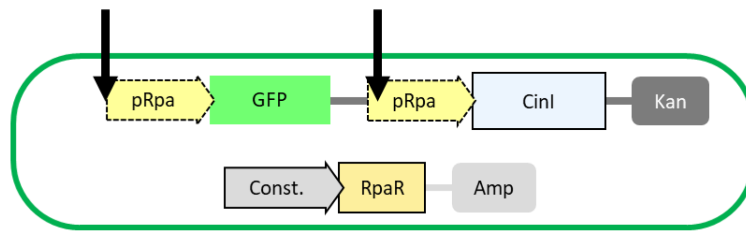


Fig 18. Signal transduction was tested with co-culture of a sender and receiver pair. The cell type illustrated with green is the sender. The sender synthesizes Cin signal (dashed line). The cell illustrated with orange is the receiver. The mKO2 expression by receiver cells, represents the signal transduction from sender to receiver. From the co-culture, fluorescent intensities of GFP (from the sender) and mKO2 (from the receiver) are plotted over time.

4.3.1. Characteristics of Co-culture

Co-cultures are good way of observing cellular communication however from co-culture data from microtiter plate doesn't show the percentage of the cells that are actively expressing fluorescent reporter. This especially difficult with co-cultures since there are two subpopulations. Therefore, we used flowcytometry to measure the percentage of cells that are turned ON (expressing a reporter). For this purpose, we tested a previously measured sender-receiver pair first individually. 95% of the sender population that contains pRpa-GFP and pRpa-CinI parts had GFP expression in culture when induced with pRpa. No GFP expression was observed without induction. (Fig 19)



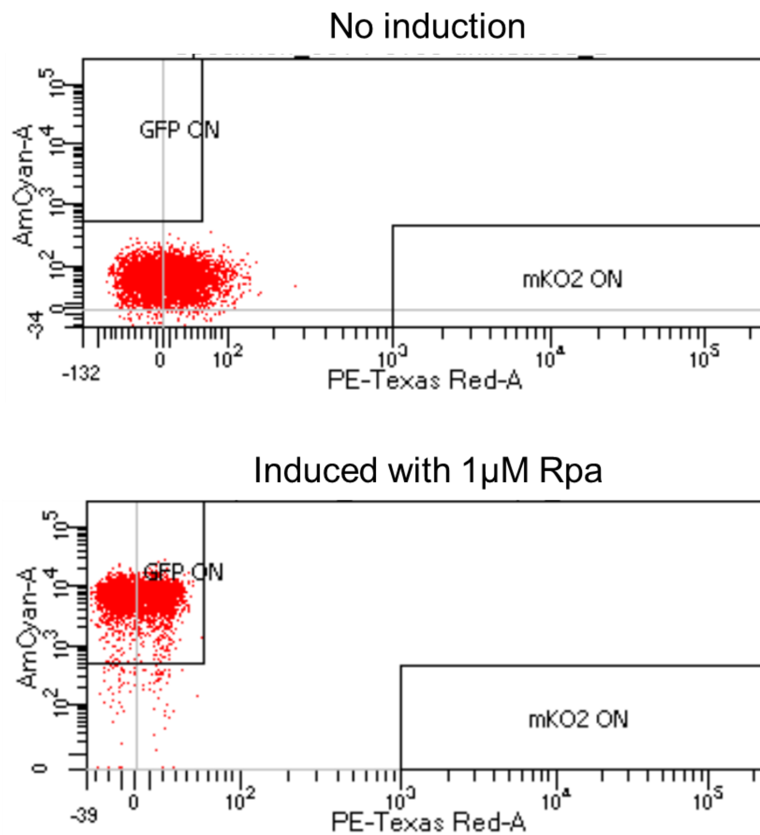


Fig 19. Fluorescent protein expression of the sender cell type in mono-culture was measured with flow cytometry. The sender that contains pRpa-GFP and pRpa-CinI parts is illustrated. The cells were detected by AmCyan filter (for GFP) and PE-Texas red (for mKO_2) filters. Uninduced and induced populations are shown in separate dot plots.

The receiver cells contain pCin-mKO2 parts. In monoculture, 99% of the receiver type cells expressed mKO_2 when induced with final concentration of 1μM Cin signal. In uninduced population, 30% of the cells had fluorescent protein mKO2 expressed (without addition of autoinducer). (Fig 20)

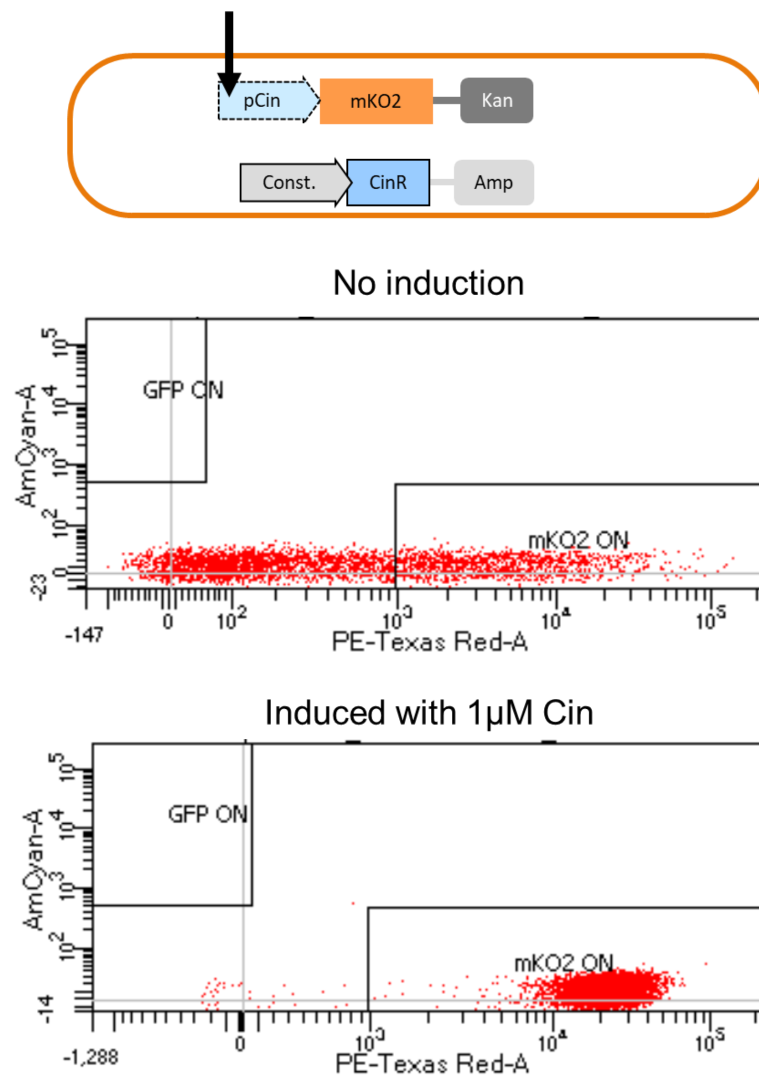


Fig 20. Fluorescent protein expression of the receiver cell type in mono-culture was measured with flow cytometry. The receiver that contains pRpa-GFP and pRpa-CinI parts is illustrated. The cells were detected by AmCyan filter (for GFP) and PE-Texas red (for mKO_2) filters. Uninduced and induced populations are shown in separate dot plots.

Following the individual measurements of the sender and receiver cells, we cultivated the sender-receiver pair in co-culture. (Fig 21) Without addition of the inducer of the sender, Rpa signal molecule, 61% percent of the co-culture had mKO2 fluorescent protein expressed. Without induction, there was no GFP expression detected. When induced with 1 μM Rpa molecule, 66.2% of the co-culture expressed GFP and 27.1% of the co-culture expressed mKO2 fluorescent protein.

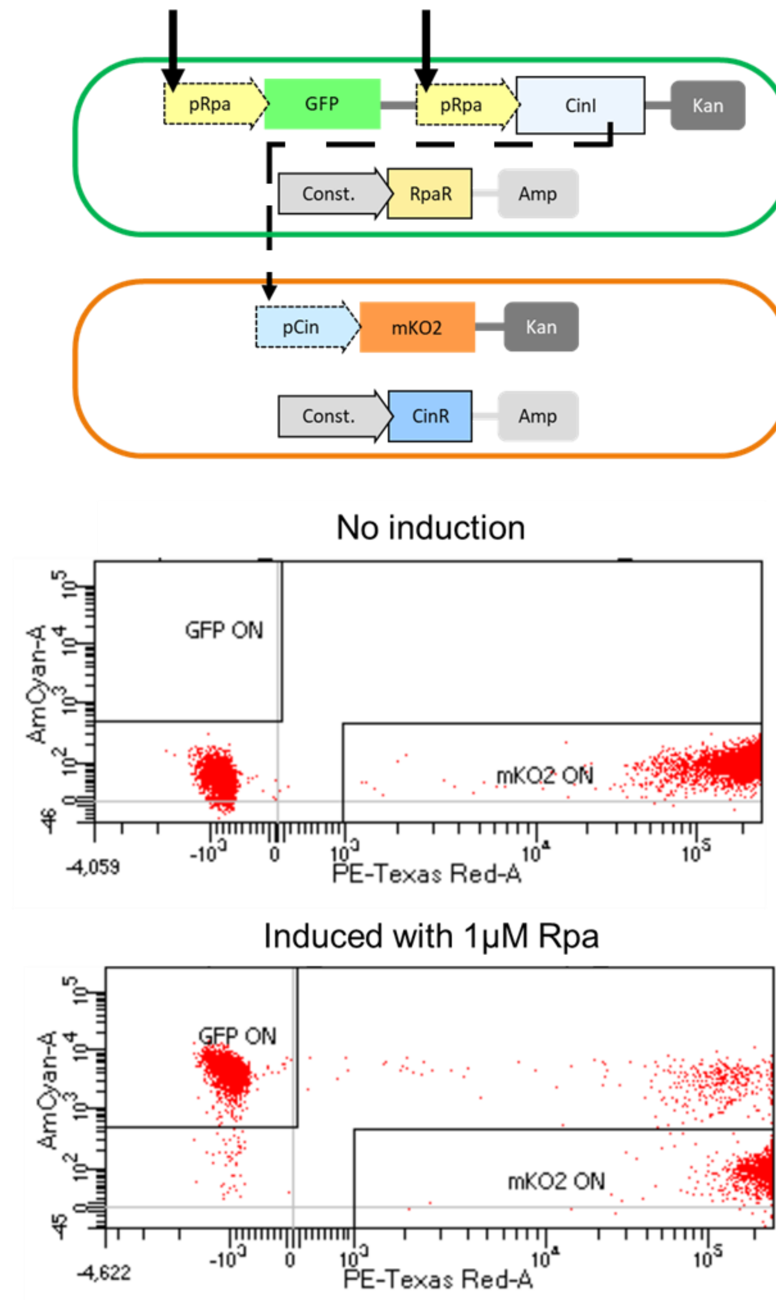


Fig 21. Fluorescent protein expression of the sender and receiver co-culture cell type was measured with flow cytometry. The sender (in green) and receiver (in orange) circuit structures are illustrated. The cells were detected by AmCyan filter (for GFP) and PE-Texas red (for mKO_2) filters. Uninduced and induced populations are shown in separate dot plots.

5. Discussion

In this study our aim was to construct a synthetic multicellular consortium in which the subpopulations exhibit cell-cell communication. For this purpose, first, we constructed a toolbox that consists of parts containing quorum sensing signaling synthases, receptors, promoters and four fluorescent proteins as reporters. From the combinations of this available elements, 15 cell types were constructed. In each cell type we transformed two plasmids: a plasmid with signal receiver and another with respective receptor promoter. We also constructed the signal generator parts with inducible promoter and quorum sensing signal synthase. Signal generator was also transformed with respective receptor backbone. To further expand the number of possible interaction topologies we included an inducible homoserine lactonase part to circuits. Addition of this part enabled an inducible OFF function via negative feedback. Thus, in each cell type there is either a signal receiver or a signal generator consisting of along with a respective receptor plasmid. Firstly, we tested the function and characterized signal receiver, receptors and signal generator parts. Then we moved on to studying signal transduction from one cell type to another in co-cultures.

5.1. Fluorescent Protein Expression

Fluorescent proteins (FP's) are widely used tools as reporters in vivo. There are numerous fluorescent proteins available. When choosing a fluorescent protein, one of the most important parameters to consider is the excitation wavelength. Ultraviolet (UV) light is known to cause DNA damage and induce formation of reactive oxygen species in live cells. Screening a fluorescent protein with excitation peak close to UV range such as blue fluorescent protein (BFB) in vivo could cause unintended mutations or impair growth of the cell line. This becomes a main problem if the cells are imaged for long periods of time with short intervals. In our case, more multiple fluorescent proteins were used to observe multiple cellular events over hours. Therefore, in addition to the to near UV toxicity, we had to choose fluorescent proteins with minimum overlap of excitation and emission spectra. When using four different fluorescent proteins there are not many ideal fluorescent protein combinations that do not overlap and away from near-UV range. We chose

eGFP, eCFP, mKO2 and mCherry as reporters for the toolbox. For the selected fluorescent proteins overlapping effect is calculated. The highest overlap is less than 2.5% in fluorescent proteins chosen for the toolbox. Based on OD600 measurements, we did not observe any growth defects that is caused by fluorescent protein toxicity.

Chosen fluorescent proteins showed significantly different intensities 3.5 hours after induction when compared under regulation of same promoter. (Fig 8) The fluorescent proteins mKO2 and mCherry, were expressed in low levels under both pCin and pRpa promoters whereas eCFP and eGFP reached significantly higher levels. This could be attributed to their folding rate since all the fluorescent proteins used in this work were monomeric. However, it is worth noting that the innate relative intensities of fluorescent proteins vary. This is important when comparing the levels of fluorescence intensities but this not a major problem since in many imaging systems the gain can be adjusted relatively. However, detection of fluorescence proteins in uninduced populations can be problematic. We observed this with mKO2. This could be due to degradation rate being slower than other proteins thus leading to accumulation of fluorescent protein expressed due to slightly leaky promoter. As the promoters of quorum sensing machinery is slightly leaky by their nature. We observed a leaky expression of mKO2 but no other fluorescent proteins in single signal generator parts. (Fig 8) pCin and pRpa promoters showed different strengths. Higher levels of fluorescent intensities were measured for all reporters under the control of pRpa promoter when both pRpa and pCin were induced with same final concentration of inducers.

5.2. Receptors and Context Dependency

We constructed three types of homoserine lactone receptor plasmids. First, single receptors contain CinR or RpaR proteins downstream of constitutive promoter. We also constructed a double receptor which contains both RpaR and CinR receptors expressed constitutively. Finally, the quencher was constructed by introduction of inducible homoserine lactonase gene (aiiA) to double receptor. Our purpose in constructing these different receptor types was to control cells by multiple quorum sensing signals. Whereas, addition of Aiia gene enabled turn OFF function. However, by comparing different re-

ceptor plasmid types, we observed that introduction of additional parts effect the performance of the cells. (Fig 11) For double receptor, this can be attributed to the metabolic burden from the constitutive expression an additional receptor. Also, addition of parts could lead to undesired affect of neighboring sequences on the expression. In the case of quencher, we observed that the uninduced cells expressed low levels of fluorescent protein unlike single and double receptors. Introduction of inducible quorum quenching enzyme lead the leaky expression. This is an example of context dependent performance of circuits. We could not have for seen the effect of context dependency prior to characterization of the parts.

The quencher part is designed to be used as an OFF switch. When tested in a circuit we observed the drop of fluorescence intensity in approximately 30 minutes following the induction of quorum quenching enzyme aiiA expression. (Fig 9) However, aiiA enzyme inactivates both quorum sensing signal, not fluorescent proteins. Thus, the effect in indirect as it inactivates its own inducer. Therefore, the rate of fluorescence intensity decrease got lower over time. There are many repressors available that work in different ways; for example, PhlF repressor can be used as an alternative to aiiA (Stanton et al. 2014).

5.3. Homoserine Lactone Synthases

5.3.1. CinI

Arac-pBad is a commonly used promoter. It is tightly activated in the presence of L-arabinose. AraC protein regulates the activity of pBad promoter and acts as a repressor. When L-arabinose interacts with AraC, it enables the activation of pBad promoter. Therefore, concentration of inducer arabinose is shown to be directly proportional to the activation of pBad thus expression of gene of interest regulated by pBad. However, for Cin HSL synthase (CinI) this was inversely proportional. Lowest concentration of supplied arabinose resulted in the highest fluorescent intensity. This result was contradictory to documentation available both in literature and in our research group for araC-pBad promoter. Thus, the experiment is repeated however result was in line with the first experiment. Hence, we do not attribute this to an experimental error.

5.3.2. RpaI

On contrary to results from araC-pBad promoter tested with CinI, RpaI paired with araC-pBad had fluorescent expression directly proportional with arabinose concentration. (Fig 13) In the absence of p-coumaric acid led to low levels of fluorescent intensity (~20 000 rfu). This could be due to unspecific activation fluorescent protein expression by the upstream promoter since *E. coli* is not able to synthesize phenolic compounds naturally. For Rpa signal synthesis, p-coumarate precursor is required (Amy L. Schaefer et al. 2008). P-coumaric acid is conjugate acid of p-coumarate, that is used in this work as substitute for p-coumarate due to its availability. However, since the concentrations used in the experiment is very low thus its affect on pH is negligible. P-coumarate is known to have anti-microbial activity (Z. Lou et al. 2012). We also observed growth problems when the culture is supplied with high concentrations of p-coumaric acid (5 and 10mM concentrations). (Fig14) 1mM p-coumarate concentration is chosen as to be optimal concentration as it did not cause growth defects but was enough for synthesis of Rpa.

5.4. Co-cultures

Following the individual characterization of cell types, we paired them as sender and receiver to characterize the signal transduction. When, the cell types are tested alone the signaling molecules that induce the circuit is administered in one dose at a certain concentration. However, in co-cultures of sender-receiver pairs, the signal is continuously produced by sender and used in receiver cell. Because of this, we observed a different dynamic in co-cultures than mono-cultures. In co-cultures, the fluorescence intensities reached to the levels reached in mono-culture with several hours of delayed.

To further investigate the dynamics of sender-receiver interactions in co-culture, we used flowcytometry. From this experiment we can extrapolate that subpopulations do not grow at the same rate even though they start at the same cell densities. This further shows that more controlled methods are vital to study dynamics synthetic consortia. This is especially important if we wish to increase the number of subpopulations in such systems for scaling up the capacity.

6. Conclusion

We constructed a toolbox with parts of quorum sensing machinery that can be used to build synthetic consortia. We characterized the parts in the toolbox alone and in pairs. In the scope of this work we could not conduct microfluidics experiments due to time limitations even though we manufactured microfluidics chips for this purpose. This work revealed that there are unpredictable factors that can affect performance of a synthetic systems including context dependency and metabolic burden on the expression host. Continuing efforts to understand cell chassis, in combination with modelling can allow us to design synthetic system in a more efficient manner in the future. Development of experimental methods that provide the controlled environment to explore complex interactions of microbial consortia is vital for this promising field to flourish.

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8. Appendices

8.1. Appendix 1

Table 2. List of the primers used

Identifier	Name	Sequence
CJ 120	CFP_Fw	AGCAGCATGACTTCTTCAAG
CJ 126	pKM_Fw	CCTATAAAAATAGGCGTATC
CJ 129	pKM_Fw	CAGAGGGCGCCCCAGCTGGC
CJ 137	RpaI_Rv	GCAGCCACCAGGTCTCCAGC
CJ 141	CinI_Rv	GAGTTCTTCCACCTCGGCGC
CJ 142	YFP_Rv	GGCCCAGGATATTGCCGTCT
CJ 177	pRpa-LR-FW3	TGTCTCTTGATCAGATCTTGATCC
CJ 261	P15-A Rv	CTGGCAGTTCCCTACTCTCGCAT
CJ 262	mKO2_Fw	TGTTCTGTTACGGCCACAGAGTAT
CJ 266	pCin_Fw	TCAGTGATAGAGATACTGAGCACA
CJ 268	mKO2_Rv	TCCGTCGCTGGCAGTAATTTTCTCG
CJ 269	CinI Fw	TCTCTACGACGTGTTCCGCGAGA
CJ 271	pKM Fw Second Insert	GCCGCTGGTTAATTAAAGCG
CJ 272	pKM RV Second Insert	CCTTTGAGTGAGCTGATACCG

8.2. Appendix 2

Table 3. Fluorescence protein excitation and emission values

Protein (Acronym)	Excitation (nm)	Emission (nm)
ECFP	439	476
EGFP	488	507
Kusabira Orange2 (mKO_2)	551	565
mCherry	587	610

8.3. Appendix 3

In this study, we constructed two sets of plasmids. The high copy number backbone with ColE1 origin of replication contains kanamycin resistance as selective marker and fluorescent proteins, eCFP, eCFP, mKO2 and mCherry downstream of pRpa and pCin promoters. For some of the constructs, Rpa and Cin HSL synthase gene, RpaI and CinI respectively, inserted under pCin and pRpa promoters downstream of the fluorescent protein.

Low copy number backbone contains with P15A origin of replication contains ampicillin resistance gene.

Plasmid maps created using, Geneious version 8.0 created by Biomatters. Available from http://www.geneious.com

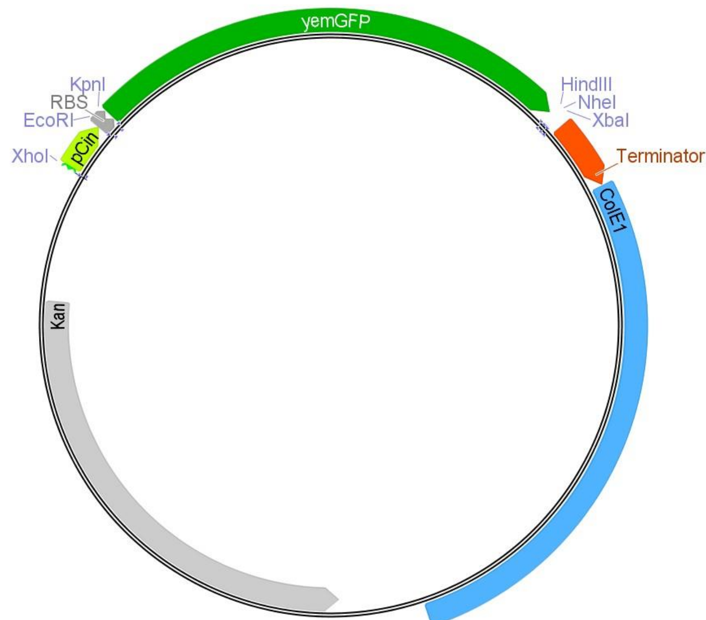

Fig 22: The plasmid map of signal receiver parts

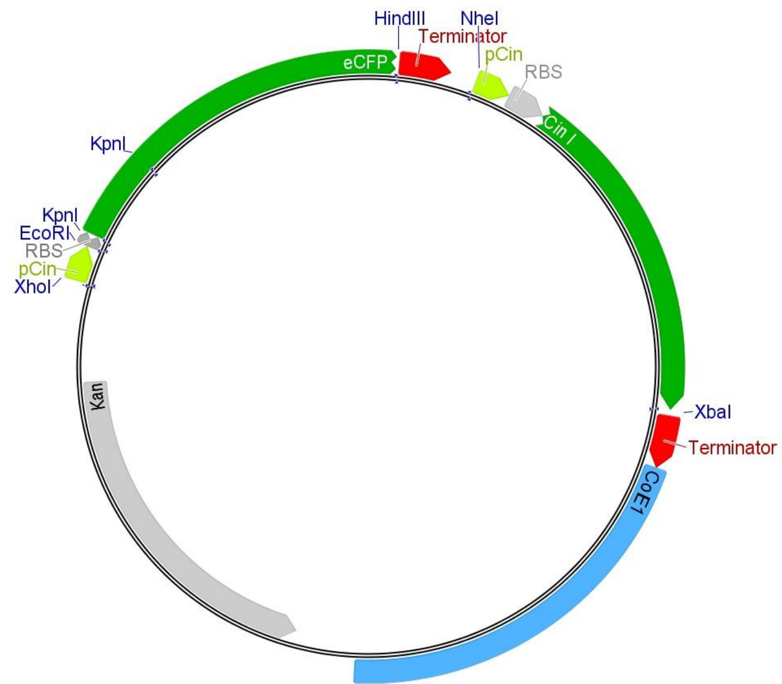

Fig 23: Plasmid map of signal generator parts

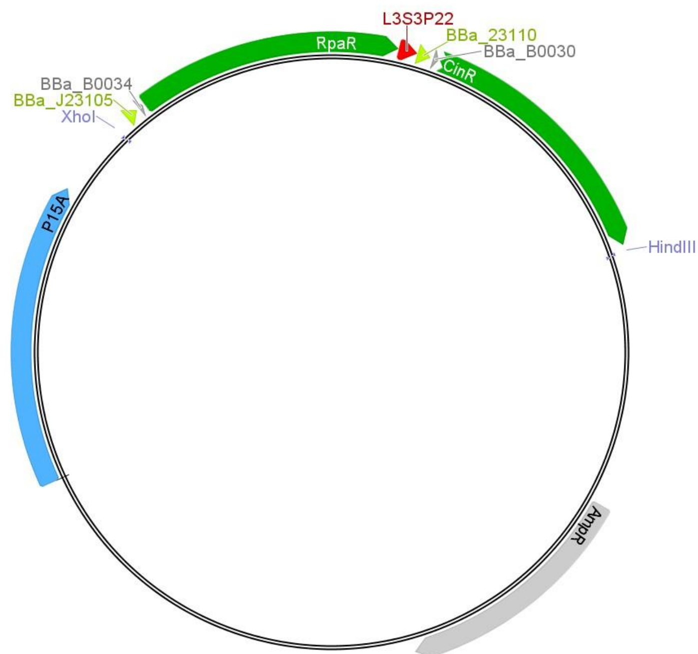

Fig 24: Map double receptor parts on plasmid

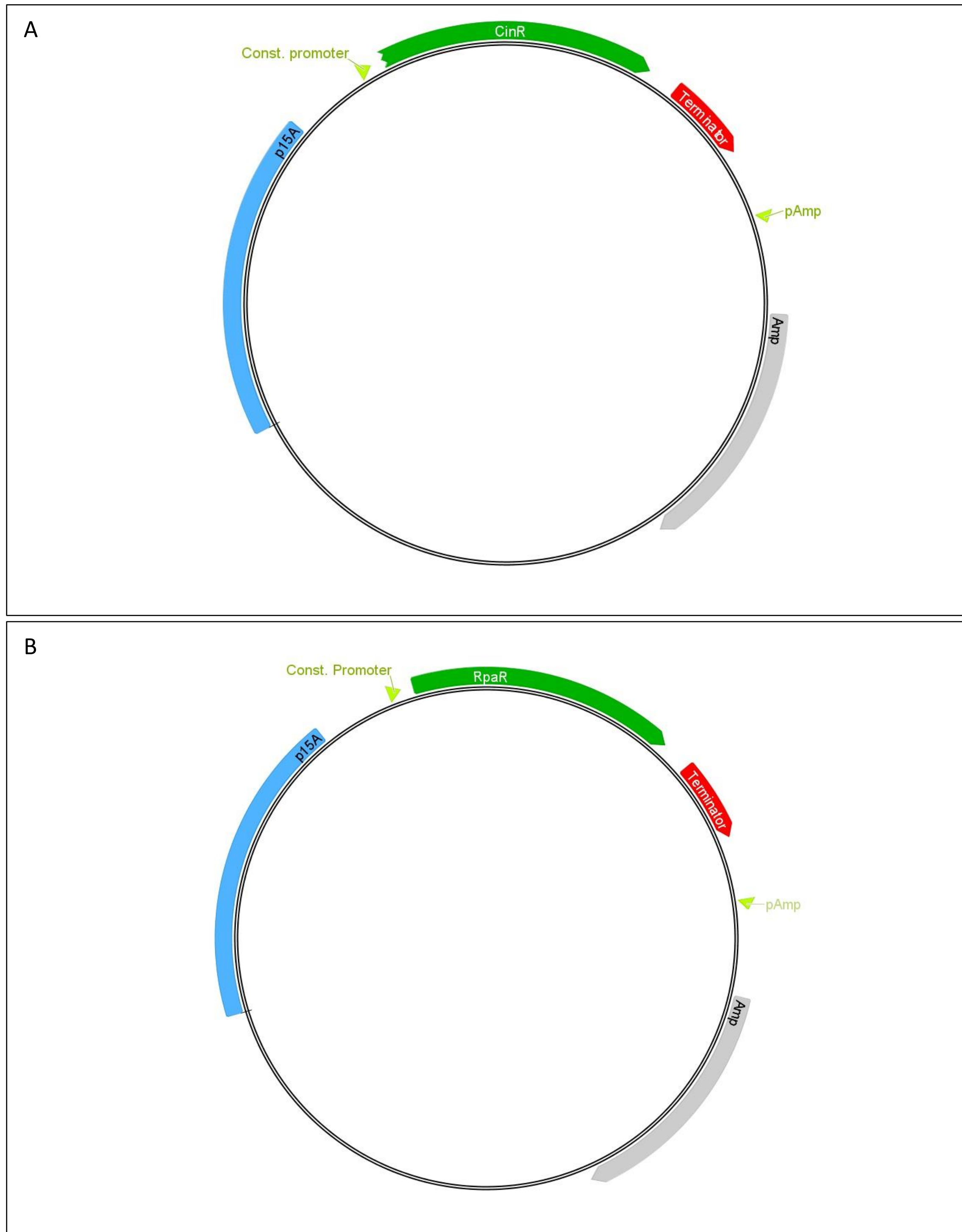

Fig 25. Map of single receptor plasmids. CinR and RpaR are both controlled by J23100 constitutive promoter (labeled as const. promoter).

Table 4. Origins of parts used

Part name	Source	Notes
L3S3P22 terminator	(Y. J. Chen et al. 2013)	
Degradation tag	BBa_M0050 (check refer-ences)	AANDENYALAA SsrA Degredation tag
pLux RBS	(Danino et al. 2010)	
(RBS of pCin*)	(Mutalik et al. 2013)	
J23100 constitutive promoter	BBa_J23100, iGEM registry	single receptor
J23105 constitutive promoter	BBa_J23105, iGEM registry	
J23110 constitutive promoter	BBa_J23100, iGEM registry	
pCin	(Y. Chen et al. 2015)	

pRpa	(Scott and Hasty 2016)	
CinR	(Y. Chen et al. 2015)	
RpaR	from *R. palustris* strain CGA009 genome	