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Co-culture systems and technologies: taking synthetic biology to the next level

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Co-culture techniques find myriad applications in biology for studying natural or synthetic interactions between cell populations. Such techniques are of great importance in synthetic biology, as multi-species cell consortia and other natural or synthetic ecology systems are widely seen to hold enormous potential for foundational research as well as novel industrial, medical and environmental applications with many proof-of-principle studies in recent years. What is needed for co-cultures to fulfil their potential? Cell–cell interactions in co-cultures are strongly influenced by the extracellular environment, which is determined by the experimental set-up, which therefore needs to be given careful consideration. An overview of existing experimental and theoretical co-culture set-ups in synthetic biology and adjacent fields is given here, and challenges and opportunities involved in such experiments are discussed. Greater focus on foundational technology developments for co-cultures is needed for many synthetic biology systems to realize their potential in both applications and answering biological questions.

1. Introduction

Co-culture systems have long been used to study the interactions between cell populations and are fundamental to cell–cell interaction studies of any kind. Recently, such systems have become of particular interest to synthetic biologists for studying and engineering complex multicellular synthetic systems. At the basic level, a co-culture is a cell cultivation set-up, in which two or more different populations of cells are grown with some degree of contact between them. The motivations for using such a set-up include: studying natural interactions between populations, improving culturing success for certain populations or establishing synthetic interactions between populations (figure 1). Examples of studying natural interactions between populations include infection studies [1], studying other natural interactions [2,3] and creating experimental models and biomimetic environments of natural systems, such as artificial tissues [4,5]. Co-cultures are highly relevant for drug research because they provide a more representative human *in vivo*-like tissue model than animal models and allow for high-throughput testing and in-depth monitoring of drug effects on cell–cell interactions [6]. Improving cultivation success is needed for certain populations. Some cells cannot easily be monocultured *in vitro* or at least do not exhibit desired *in vivo* physiological behaviours [6–11], but the presence of another cell population may improve the culturing success or cell behaviour [12]. Establishing synthetic interactions between populations is becoming more and more common. While mixed cultures have long been used for industrial applications, there has been a strong drive in synthetic biology and other biological fields to develop complex systems with industrial applications. Many of these require co-culture systems. Examples include synthetic industrial consortia [13–21], synthetic ecologies [3,22–25] or other complex interactions [24,26]. Synthetic ecologies are widely seen to hold great potential for fundamental research as well as industrial, medical and environmental applications [6,19,27], as they have been shown to have increased productivity and other advantages over monocultures [28,29].

Co-culture systems will be central to the synthetic biology progress. Studying natural cell–cell interactions will highlight new pathways for re-engineering even in difficult to culture organisms. The ultimate aim of synthetic biology is to

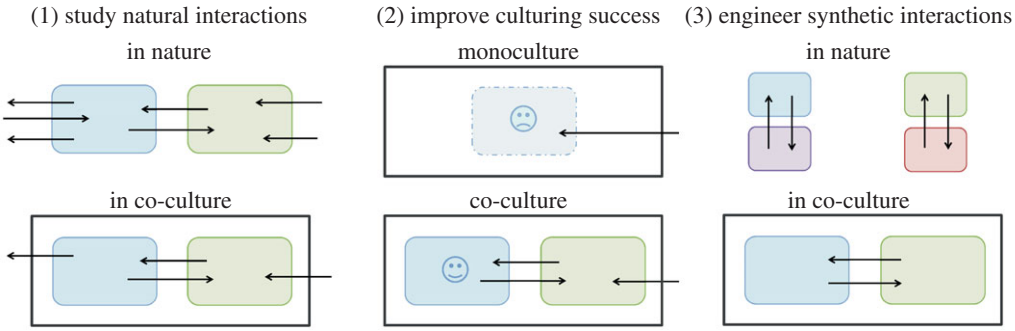

Figure 1. Co-culture definition and motivation. Main reasons for conducting co-culture experiments. (1) Studying natural interactions between populations. (2) Improving cultivation success for certain populations. (3) Establishing synthetic interactions between populations.

deliver societal benefit in industrial, medical and environmental applications [30,31]. Therefore, many synthetic biology systems are developed with future industrial, medical or environmental co-culture applications in mind [30,31], such as cell–cell communication [20,26,32], toggle switches for population control [33] and bacterial cells engineered for killing pathogens [34], tumour targeting [35,36] or therapeutic delivery [37]. Before these systems can be used in industrial, environmental or medical contexts, they have to undergo rigorous testing for function and safety, especially in terms of the cell–cell interactions [34,36] and subsequent process optimization. Co-culture systems as model systems of tissues or ecologies are very complex with interactions happening at different length/time scales [2]. Experimental systems are required for these studies allowing for monitoring of complex cell consortia in a high-throughput manner, large-scale data collection, as well as control and variation of experimental parameters.

Synthetic biology is an inherently interdisciplinary field and this is reflected in the technology used. Current co-culture techniques were originally developed in a number of fields, including mammalian cell studies for medical applications and microbial ecology. Owing to these diverse interdisciplinary origins, there is no unified terminology for such experiments and the same term may mean different things in different fields [16,17]; common terms include coculture, co-culture, binary culture, heteroculture, heterotypic culture, mixed culture, dual culture, bi-culture, tri-cultivation, co-localization or co-cultivation. This makes the availability of co-culture technologies somewhat unclear. This review aims to provide an overview of currently used technologies for co-cultures between cell populations and highlight the gaps in current technologies, which may serve as bottlenecks for co-culture systems to reach their full potential in synthetic biology applications.

1.1. Extracellular environment: a tunable dial

The main motivation for conducting co-culture experiments is to study cell–cell interactions of any kind, both natural and synthetic, or to engineer new such interactions. Such population interactions are strongly affected by the extracellular environment, which in turn is determined by the experimental set-up. Therefore, it is necessary to develop experimental systems that are capable of replicating the environment in which the co-culture will ultimately be used. Cellular phenotype is produced by complex interactions between genotype and environment. For co-culture studies, this means that the environment not only interacts with the (natural or synthetic) genotype of each cell population to affect behaviours, but the structure of the population environment can fundamentally change the way in which populations interact and whether the interactions are stable [38]. For example, populations may form cooperative relationships under some environmental conditions and not others [3,23] and the behaviour of cell populations may change if cultured in different vessels [39]. This long-established principle in ecology has also recently been experimentally demonstrated in a number of synthetic ecology experiments, both in the wet laboratory [10,22,40] and *in silico* [41,42]. While control of the extracellular environment is always important, the situation becomes more complicated in co-cultures, where several distinct or semi-distinct environments may need to be established and controlled. In general, synthetic biologists aim to control the behaviour of cell populations through genetic manipulation. Alternatively, interactions in co-cultures can be stabilized by varying the environment over time [43–45]. In addition, the effect of the environment on the evolutionary trajectory of co-cultured populations requires careful consideration, ideally at the stage of gene circuit design [46], as mutants are more likely to arise in large cell populations [20] or when cells are under a high degree of stress. This is both a challenge and an opportunity. As synthetic gene circuits only work as expected in some environments they need to be designed for and characterized in the intended working environments. On the other hand, the extracellular environment can be used alongside genetic modification as an additional tool to control cellular behaviour, e.g. to induce cooperative behaviour by choosing a structured Petri dish environment over a well-mixed flask [10]. Therefore, experimental conditions and choice of equipment for co-culture experiments need to be carefully considered.

2. Variables in co-cultures

Existing co-culture systems differ in many variables, some of which will be discussed here. Although these variables are separated here for clarity, they are all interlinked in their effects on cell behaviour in co-cultures. Co-cultures can differ in terms of the number of distinct co-cultured populations, degree of similarity between populations, degree of separation between populations, difference between population local environments, volume of cultures and time scale of co-culture. There exist trade-offs between these factors. For instance, specialized separations between cultures have most successfully been done at the small volume scale and with small numbers of distinct cultures.

2.1. Number of populations: complexity issues

Synthetic ecosystems follow the same trend as other levels of the synthetic biology hierarchy such as modules and

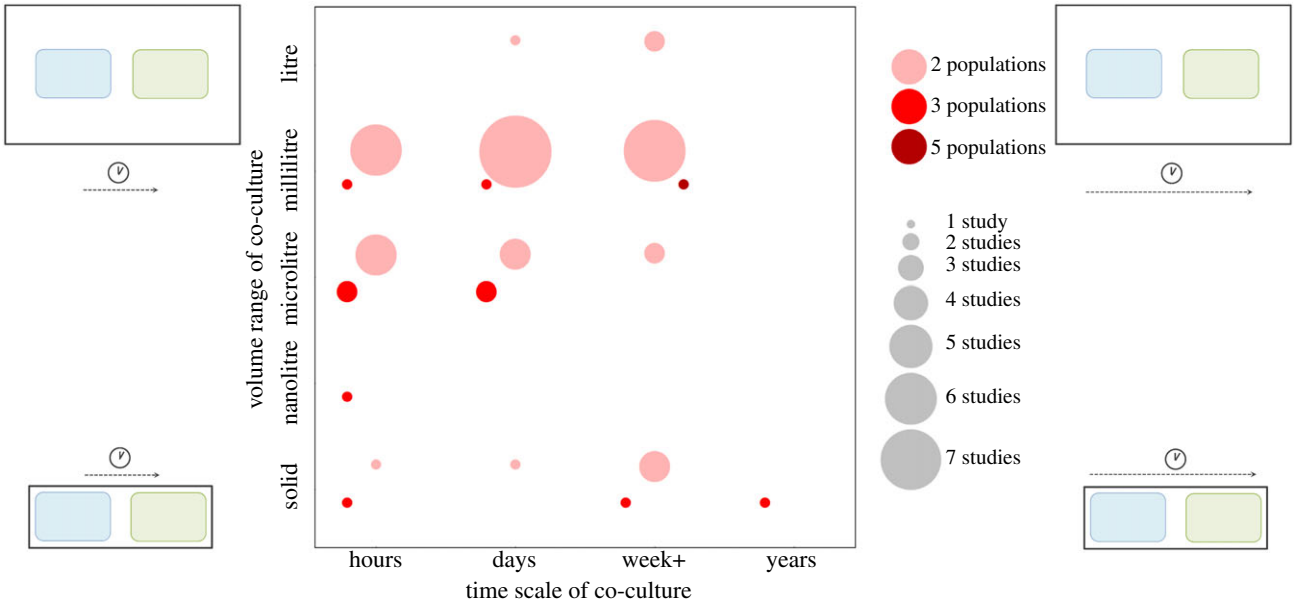

Figure 2. Overview of co-culture studies in terms of time scale, volume and population number. Data based on 47 published co-culture studies. For studies presenting multiple co-culture systems, the most 'extreme' one was chosen. If a study compared different co-culture systems, the most successful one (in terms of cell growth) was chosen.

intracellular networks in terms of what level of complexity has been achieved [29]. Many systems involving only a small number of genes and regulatory elements exist, but there are few systems that are based on a large number of genes. Similarly in co-cultures, a large number of lower complexity systems have been established, but examples of higher complexity systems remain few and far between and beyond a certain complexity level no defined systems have been established (figure 2). The reasons for this are manifold and have been previously discussed [47]. A larger number of interacting populations increase the possibilities of complex reaction pathways with industrial applications, but also vastly complicates the experimental procedure. More than three interacting cell populations can lead to a currently unmanageable level of complexity in terms of the molecular interactions, leading to unpredictability and instability [18]. The vast majority of co-culture studies to date involve two populations, owing to these issues of complexity in establishing a stable system when more populations are involved (figure 2).

A smaller number of these have studied the interactions between three cell populations, including defined synthetic ecologies [11,22,36,40,48–52] and organ model systems [53]. Studies on co-culturing more than three distinct cell populations are rare, but include small-scale *in vitro* tissue models [4,54] or less defined consortia with industrially promising functions [55]. In order to help deal with the complexity arising from a natural consortium, Kato *et al*. [55] created a synthetic ecosystem of five co-cultivated bacterial strains as a cellulose-degrading consortium by selecting the most dominant species from the natural environment. Culturing was done under static conditions, which may have aided stable co-culture of this large number of species (see §2.2).

However, in many situations, it may be possible to avoid the problem of complexity altogether since some studies have suggested a larger number of populations may not always be advantageous. For example, Hatherell *et al*. [56] compared mono-, bi- and tri-cultures as models of the blood–brain barrier and found that a bi-cultivation performed best. This is in line with the general goal of synthetic biology to engineer systems that are only as complex as necessary to achieve the desired goal.

There are significant challenges in engineering and designing synthetic ecosystems with predictable outcomes, although studies such as the human microbiome project (http://hmpdacc.org/) could provide insights into highly complex microbial ecosystems of medical relevance that could be used for other applications. However, the abstraction hierarchy that synthetic biologists use for designing complex systems could provide a useful framework for considering synthetic consortia as it would allow high-level design decisions to be considered without reference to genetic level manipulations.

2.2. Large differences between cultured populations enabled by set-up

The cell populations in a co-culture may be very similar to each other, e.g. the same strain only differing in the expression of a gene, or they may be very different from each other, e.g. different species. The difference between the co-culture populations can also be defining for their ecological relationship, such as competitors, predator and prey, parasite and host or cooperators.

While it seems intuitive that the more similar the populations being co-cultured are the easier it would be to establish a stable system, there are many successful examples of co-cultures involving vastly different species. The co-culture of very different species is often enabled by the experimental set-up, e.g. by introducing separate compartments that allow individualized environments and minimize the potential for inhibitory contact.

For same-species co-cultures, examples in the literature include co-cultures of two mammalian cell populations differing only in a fluorescent label [57,58], co-cultures between populations of the same species that differ either through natural specialization into strains [40,59,60] or through genetic modification [11,12,28,40,48,49,52,61–68] or co-cultures between mammalian cells from different tissues of the same species [52,53,56,69–76]. The last are often the first step towards artificial tissues and organs. Co-culture of different strains simulates

natural ecosystems and can be used to test evolutionary principles. By co-culturing differently engineered strains, it is possible to build up complex behaviours, such as pattern formation or symbioses.

Examples of co-cultures of more diverse populations include cultures of cells from different species of multicellular eukaryotic organisms [77–83], fungi [84] or different bacterial species [10,18,22,34,55,85,86]. Mammalian and bacterial cells have been co-cultured, either for infection studies [1,87,88] or testing of synthetic bacteria [35,36,89]. Plant–bacterial and fungi–bacterial combinations have also been co-cultivated [43,90]. Some of the examples include very diverse species grown in close proximity. For example, Lõrincz *et al*. [51] studied an artificial symbiosis between a photosynthetic green alga, a nitrogen-fixing bacterium and a fungus grown on carbon- and nitrogen-free medium. Although this synthetic community was discovered serendipitously, it shows that co-cultures require similar conditions to be stable over generations as natural symbioses. All three organisms contributed positively to the symbiosis. The bacterium provided nitrogen, the alga provided carbon and the fungus is thought to provide additional compounds. In another example, Weber *et al*. [49] engineered mammalian receiver cells (CHO cells) to receive communication from another (sender) population. For the sender population, they variably used a diverse set of cells, including CHO cells, HEK293-T (human embryonic kidney) cells, *Escherichia coli*, *Saccharomyces cerevisiae* or *Lepidium sativum* (garden cress). In this case, inter-population communication was via volatile substances, thus enabling sender and receiver populations to be kept in separate compartments. This allowed for very different growth conditions for each population and hence made co-culture of such vastly different organisms possible.

2.3. Degree of contact between populations: physical boundaries allow more individualized environments

Depending on the experimental set-up, the populations could be perfectly mixed or partially separated. In general, choosing a particular mode by which to separate the cell populations allows control of the population interactions, which can be key to achieving a stable system. The method of population separation needs to be carefully chosen to ensure relevance to the ultimate application of the co-culture. For example, if the populations are dependent on each other for substance exchange, permeability of materials must be considered because diffusion rates within specific ranges may be required (e.g. [49]). When diffusion rates are too low, important nutrients cannot be exchanged. But it is also possible for diffusion rates to be too high; for instance, if one population secretes substances that are toxic to another population, as in the predator–prey system by Payne *et al*. [91]. These factors need to be taken into consideration when scaling up co-cultures to greater volumes as diffusion is a distance-dependent phenomenon.

For some applications, the different cell populations must be in direct contact, sometimes called mixed culture. Direct contact is often required in mammalian tissue and other eukaryotic cell cultures to preserve physiological behaviour [53,66,74,83]. Similarly, infection and invasion assays also require direct contact because of the aim of the study [1,35,87,88]. In mixed co-cultures, the different populations are kept under symmetrical conditions, which is possible when the two populations are very similar to each other in terms of their requirements or when using rich growth media. If the populations are different from one another, but a mixed culture is desired, growth medium optimization is probably required to select the medium that best sustains all the cell populations [41,66]. Alternatively, it may be possible for populations to be adapted to conditions that allow symmetrical culture [88]. There is often a need to grow populations separately under asymmetric conditions before inoculating the co-culture.

In other studies, contact between cells is allowed, but carefully controlled through techniques, such as cell immobilization [82], microfluidics [6,58,73] or cell micropatterning [54,71,72,77,78,81,92] (figure 3). The type of micropattern used can strongly affect the culture fate [72,77], because it will determine diffusion rates and level of separation or contact between cell populations. Owing to cell polarization, exact positioning may be needed for mammalian cell studies to ensure that population contact is made via the correct cell surface [88]. Williams & Wick [70] developed a vascular construct perfusion bioreactor that allows structured biomimetic contact between two cell populations. Albrecht *et al*. [57] showed three-dimensional micropatterning of mammalian cells in hydrogels using dielecrophoretic forces. These techniques could be adapted to create a more stable configuration for a synthetic co-culture for other purposes or to create synthetic tissue mimics with a similar structure to natural tissues.

In cell migration assays, populations may be separated through either a physical barrier or simply a gap, which is subsequently removed [76]. In some cases, separation between populations does not need to be enforced, as the different niches of the two populations naturally lead to some degree of physical separation. For example, co-cultures where one population is adherent to a solid support and the other is in suspension [1], or one population grows in liquid medium and the other at the medium–air interface [59]. Generally, any difference in local environments even as simple as a concentration gradient [6] can potentially cause cells to separate. This happens in natural environments, for example between the aerobic and anaerobic zones of water [16]. Therefore, non-uniformity, which is undesirable in monoculture reactors, could be used to enhance co-culture success, and separation allowing local ecological processes can stabilize ecosystems compared with environments with large-scale mixing [22,23,40].

Cell populations that communicate through diffusible molecules may be separated to a larger degree without disrupting communication. This can enable the culture of vastly different cell types with different growth requirements. With increasing degree of separation comes the possibility of having different local environments for the populations. This can be used to optimize growth conditions for each individual culture (especially if the growth requirements or growth rates of the populations differ substantially), to expose populations to different environmental factors or to promote and stabilize certain ecological interactions [40,93]. By separating populations, co-culture systems can be treated as comprising ‘monoculture modules’ that can be connected to other such modules in a defined way [29]. This is a direct analogy to other aspects of synthetic biology which seek to build systems up by interconnection of smaller, well-characterized components.

Separation can be achieved by semi-permeable membranes [12,22,67,69,75,80], gels [36,79] or using microfluidic

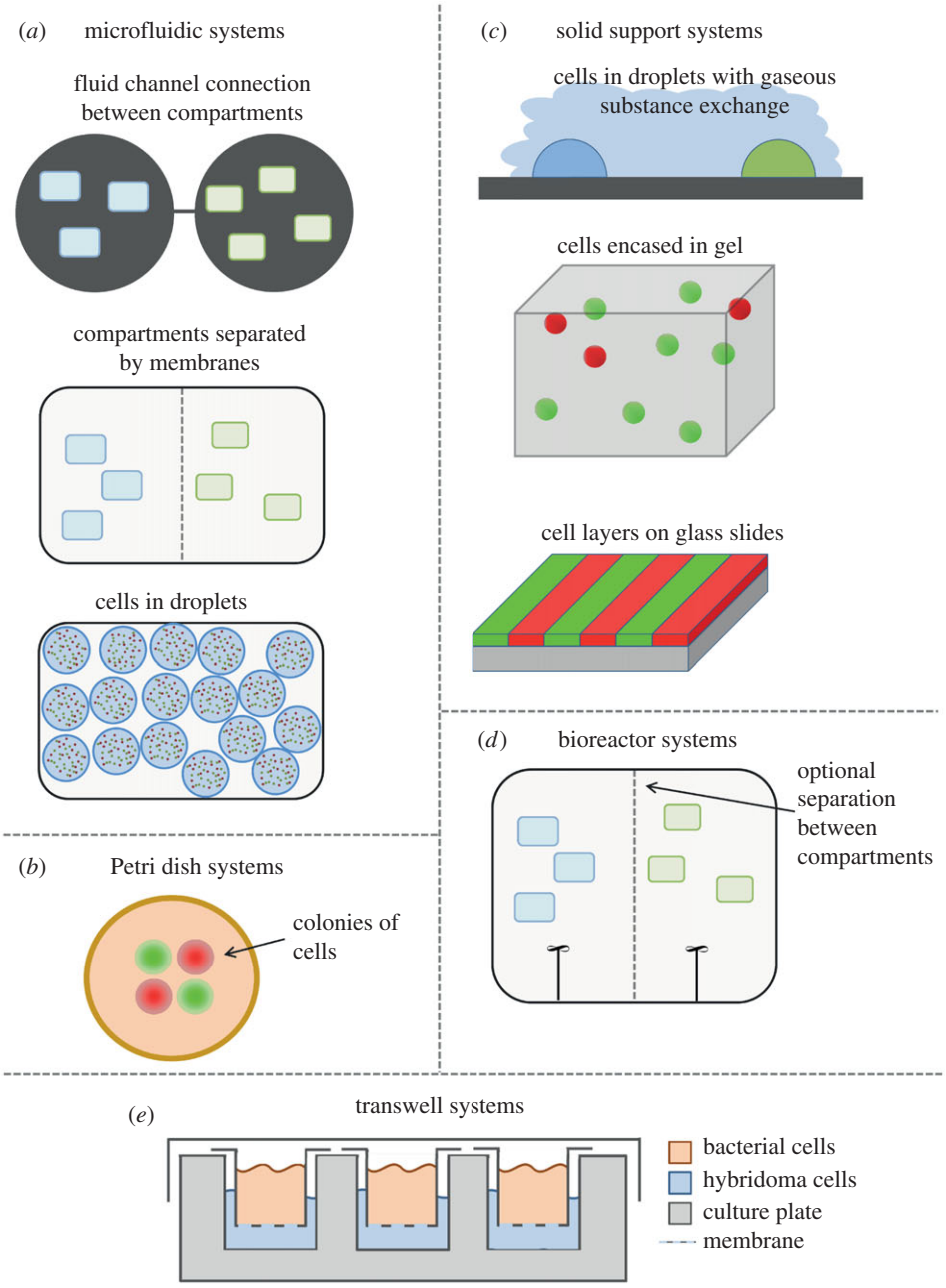

Figure 3. Selection of existing co-culture systems by type of technology used. (*a*) Microfluidic systems using fluid channel separations between populations [36,58,79], membrane separations [1,12,22] or droplets [11]. (*b*) Petri dish co-culture systems (e.g. [40]). (*c*) Co-cultures on solid supports. Using gels [57], droplets [68], microarrays [82] or cell layers on glass slides [81]. (*d*) Co-culture systems using bioreactors, with possibility for separation between population compartments [17,67,70]. (*e*) Transwell systems for co-cultures. Allowing for asymmetric levels of separation between various numbers of populations [52,56,74].

devices. Possible separation methods in these platforms have been previously reviewed [4,6,94] and range from laminar flow to hydrogels. Populations can also be separated through different liquid phases. Byun *et al*. [68] created microcolonies in single droplets in which a sender colony communicated with a receiver colony. The droplets contained magnetic particles and could be moved across the surface for precise positioning (figure 3). Weber *et al*. [49] separated populations even more, through culturing populations in separate plate wells with communication happening through volatile substances that travel via the air.

If there are more than two populations, then there is the potential for different kinds of separations between the populations, allowing each population or pairs of populations to be connected to other populations in a defined way [56]. Bacchus *et al*. [52] designed artificial two-way communication between two HEK cell lines (in a shared culture compartment) and used this in transwells to control an endothelial cell line (in a second compartment separated by a membrane; figure 3). Asymmetric separations between populations can be used to allow one-way communication. Hong *et al*. [36] constructed a microfluidic device for investigation of bacterial cancer targeting, which could be used to create one-way communication from the cancer cells to the bacterial cells. Whether communication between populations can be unidirectional or bi-directional directly influences the types of possible interactions [29].

The effect of separation distance between populations on cell behaviour is most clearly demonstrated in co-cultures for pattern formation, as different levels of separation lead to different visual outputs [48], but the principle is equally important in other cultures even when it cannot be visualized.

Different degrees of separation between the populations in a co-culture bring both advantages and challenges. Separation of populations can be difficult to set up, but allows control and monitoring of interpopulation interactions, which can be useful when troubleshooting system design. Complete mixing of populations complicates measurements of both intra- and interpopulation interactions. Cell patterning systems and other methods of population separation are very promising for structuring cell populations in small-scale bench experiments, but are not always suitable for large-scale industrial processes owing to cost and incompatibility with product purification processes [21,95]. In cases where separating populations is not a viable option, genetic control for population structure or time-variant conditions could be used [21,43–45]. Varying the conditions over time can favour different populations in turn, thus allowing all populations to survive in the long term. Genetic manipulation can make populations dependent on one another for nutrients, which will prevent one type of cell from outgrowing the others.

2.4. Volume of co-cultures: reporting standards to enhance progress

Co-cultures could potentially be carried out at any volume range, and it is the application that most probably dictates the choice of volume. For instance, culture volume and cell density can be important factors when establishing biomimetic *in vitro* models. In a study investigating the embryonic developmental processes segregation and pattern formation, cell number in the co-culture was chosen to be comparable in size to an embryo, so that it was relevant to the natural biological system [83].

In biotechnology, there are two opposing forces in the scaling of experimental volumes: efforts to scale up experiments to industrial volumes and yields, and efforts to scale down experiments to the microlitre scale to allow high-throughput testing of different systems or conditions and also for point-of-care or in-the-field applications [96]. The volume, i.e. reactor capacity, will (alongside with other factors) determine the possible population sizes in the co-culture. It is well known in biotechnology that, when scaling systems to larger or smaller volumes, it is impossible to keep all hydrodynamic properties constant. Thus, it will be likely that when scaling co-culture systems different criteria will need to be developed to ensure a stable system can be achieved.

Small-scale, high-throughput systems will be useful for identifying a preliminary set of conditions to investigate further for a desired system. For these systems, keeping reagent volume usage to a minimum is useful to reduce costs. As most common pieces of laboratory culturing equipment have volumes in the millilitre range, many reported co-cultures are in this volume range and have used microfluidic devices [12,22,36,53,58, 62,73,76,79], 96-well plates [49,64,88] or microdroplets on Petri dishes [68]. However, systems with even smaller volumes have been developed. For example, a co-culture system was reported using microbial communities encapsulated in microdroplets of size approximately 1 nl allowing high-throughput testing [11]. In an extreme example of downscaling, Frimat *et al.* [58] built a microfluidic device that allows single cell co-culture used to investigate cell–cell contact between two mammalian cell lines, which would allow even further increases in throughput in screening experiments.

On the other hand, when moving to production, larger volumes will be necessary in order to satisfy production requirements. Co-cultures in the litre range using bioreactors have been reported [18,52,85]. Co-cultures have also been performed on solid media, such as Petri dishes [10,28,34,40,48,83], slides [71,77,78,81,82] or within cell patterning chambers encased in hydrogel [57]. Asymmetric culture volumes for the two populations are also possible [80,87]. However, as volume increases, maintaining a well-mixed environment becomes more difficult, leading to the potential for heterogeneity within the system. This could cause instability in the system Reactor volume can affect co-culture viability [61]. Biliouris *et al.* [42] present an extensive stochastic model of a synthetic bacteria–yeast ecosystem and simulate reactor capacities, seeding densities and seeding ratios that would support the proposed system. For this system, population trajectories become more variable in larger reactors, leading to a less predictable system. Population size control can be used here (see §2.5). However, to date, culture volumes in co-culture experiments are not always reported in sufficient detail. This inhibits reproducibility and scale up. It is expected that co-cultures will become ever more prominent in synthetic biology and biotechnology and that standard practices for the level of experimental detail reported will arise, as has been adopted in other areas of synthetic biology (e.g. characterization datasheets).

2.5. Population control: improving overall culture success

Related to reactor volume and the growth rates of different cell types, population sizes in a co-culture often differ vastly with one being the dominant population. To look at it a different way, in co-cultures the population ratios often have to be optimized to obtain a stable culture so that one cell type does not eliminate the others [66,97]. In mammalian cell co-cultures, the population ratio is commonly set to a value resembling the natural situation. For synthetic interactions, various ratios will need to be tested to find an optimal one. Different co-culture studies have different aims and with those come different requirements for population control. For example, if the aim for a co-culture is to make a stable or oscillating system of several populations, the growth of some populations may need to be suppressed. However, if experiments are being carried out for a short amount of time, then these considerations may be less important. In industrial settings, natural consortia may be subjected to selection and screening processes to establish reduced, more efficient and more defined consortia for subsequent use [15]. Therefore, in some co-culture applications the aim may be to eliminate rather than stabilize certain populations. Population control and stabilization has previously been discussed [3,20,29].

Population densities and ratios can be enforced in a number of ways: through the use of selectively toxic compounds, genetic engineering or reactor design, the choice of which is context-dependent. While coexistence of bacterial and mammalian cells is necessary during infection studies, bacteriostatic compounds can be used to limit the bacterial population density in order to allow monitoring of cell–cell interactions without elimination of the mammalian cells [87]. Genetic engineering can be used to manipulate population ratios by defining the growth rate ratio. In one example, a cell population was made auxotrophic such that the medium

concentration of the required compound would define the growth rate of that population [64]. Predator and prey populations naturally control one another's growth rates [62], and synthetic systems have been made in which the populations control one another's growth through communication [42]. Natural mechanisms exist that prevent the depletion of nutrients by some cell types [60]. Periodically varying co-culture conditions, such as dilution rate or pH, can be used to differentially manipulate population growth rates, allowing coexistence of populations with different growth rates that would not be possible under time-invariant conditions [43–45].

2.6. Time scale of co-culture experiments: unexplored parameter space

Co-culture model systems of tissues or ecologies are very complex with interactions happening at different length and time scales [2]. If the aim is to study the interactions between cell types, then time scales of minutes to hours may be sufficient and there is no need for the co-culture to be stable in the long term. However, for industrial production, longer time scales may be required, and stability is necessary to ensure consistent production. In this case, testing may be required to ensure that materials used in cell cultures are stable in the long term [71]. If a particular evolutionary trajectory is required (e.g. all populations survive), then the situation becomes more complicated and requires further optimization. Long-time-scale cultures require continuous culture-type conditions such as nutrient feed and waste removal and are therefore only possible in set-ups that enable this. For example, Shou *et al*. [61] present experiments into the long-term behaviour of a co-culture. Using daily dilution of the culture, cultures were grown for up to 500 h. Over the course of the culture, cell populations became more tolerant in their cell density requirements possibly owing to changes in the strains. This shows that cell populations in long co-cultures can be subjected to natural selection and evolution. This can make the co-culture more stable, but also less predictable. At small volumes, microfluidics platforms have been developed that allow continuous culture for weeks using a strategy equivalent to a nutrient feed adapted to small volumes [94]. For instance, in a co-culture using droplet microcolonies, periodic relocation of droplets circumvented local oxygen depletion allowing stable culture for longer [68]. Some level of nutrient feed and waste removal is probably needed for co-cultures at all volume scales.

However, in some cases, details of co-culture time scale are not reported at all. As with the culture volume, adopting better reporting practices for metadata such as this will be useful for determining what the limits of different co-culture experiments are. Towards this end, experiments should be run for longer than just what is necessary for data collection for the immediate questions since, for many applications, it would be useful if cultures were stable for long times.

3. Models of co-cultures to enable design

An important aim of synthetic biology is to engineer systems with predictable behaviour. A vital part of this is to be able to computationally model these systems. One of the major challenges of modelling co-culture systems is that they are inherently very complex because they comprise several populations. It has been stated that the long-term behaviour of such systems is unpredictable [14], although it was subsequently suggested that evolutionary and ecological theory could be used to predict long-term behaviour accurately [38,46]. Some modelling considerations for synthetic co-cultures have been discussed previously [17,25,29], and there are numerous published models of co-culture systems [18,65,80]. The focus here will be on models that relate to experimental conditions in co-cultures and hence assist choice of equipment. A summary of co-culture factors that are commonly modelled is shown in figure 4.

The factors that are most important in co-culture (population dynamics and interactions) are often not considered in single culture models, making it difficult to translate current single culture models into co-culture models [17]. However, there have been recent publications showing complete theoretical or theoretical–experimental approaches for establishing co-cultures. Hanly *et al*. [98] aimed to design a microbial co-culture that exhausts glucose and xylose simultaneously by steadily increasing complexity from monocultures under ideal conditions to monocultures under co-culture conditions and then to actual co-cultures with modelling predictions at each step. This approach shows that co-cultures are more feasible with organisms that have been extensively characterized both experimentally and theoretically in isolation. Schmidt *et al*. [99] report a workflow for finding unknown interspecies effects in a mixed culture by modelling the co-culture based on data from monocultures, and then using experimental deviations from model predictions to identify unexpected species interactions. Dalchau *et al*. [39] report a model of the experimental system by Balagaddé *et al*. [62] using the genetic engineering of cells language in which important environmental factors for the system are derived [39]. The authors simulated the effects of parameters that can be easily tested in the laboratory, at least at a low-throughput level: effect of pH buffer (can change population behaviour if cell communication molecules are pH-sensitive), effects of different diffusion rates and population starting patterns, making this a useful tool for system design. Any assumptions made are carefully considered in terms of whether they hold true for the environment in which the system will be tested. The approach can be used to see whether a chosen genetic circuit will give desired population behaviour under any environmental conditions or if redesign is necessary. Originally, the system was tested under well-mixed conditions, but simulations show that the system would have worked differently under static conditions. The results suggested the need for certain genetic parts that had not yet been engineered, specifically stronger ribosome-binding sites. The approach can be used to see whether a chosen genetic circuit will give desired population behaviour under any environmental conditions or if redesign is necessary.

The most commonly modelled factors are those for which it is difficult to experimentally explore a significant portion of parameter space: population ratios, environment structure and long-term behaviour. Kerr *et al*. [40] show experimentally and theoretically that ecosystem diversity increases in mixed compared with static environments. Byun *et al*. [68] show how a co-culture interaction is negatively affected by local oxygen depletion and show that this can be overcome by colony relocation. Kim *et al*. [22] presented a tri-culture system and a model defining three possible classes of mixed culture communities. The results from this model could be used to plan suitable experimental set-ups for other co-cultures. Biliouris *et al*. [42] present an extensive stochastic model of a synthetic ecosystem which accounts for intrinsic

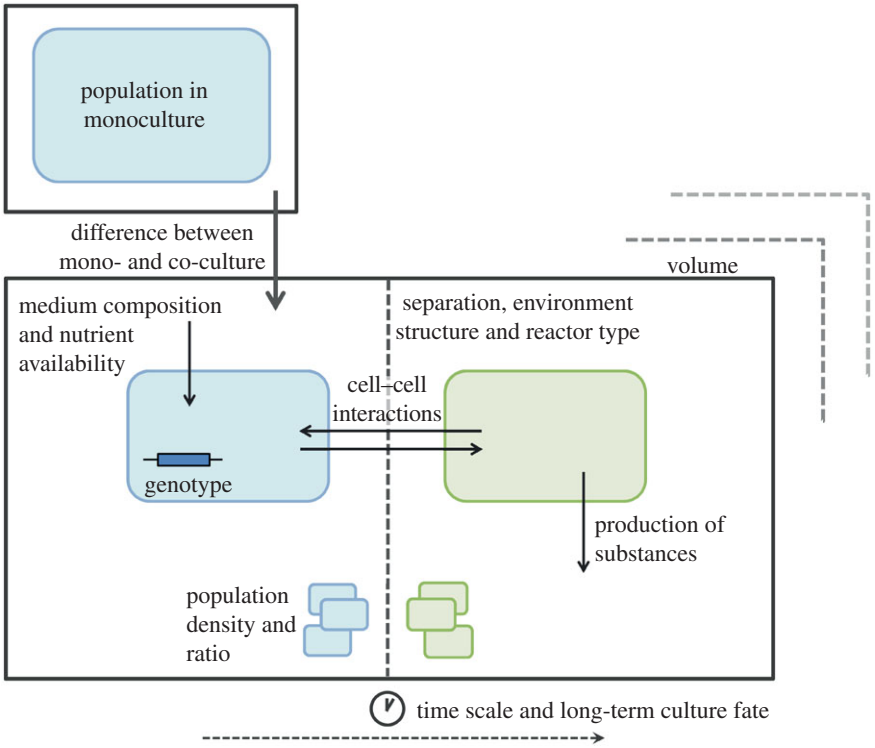

Figure 4. Co-culture factors commonly investigated using mathematical modelling. Based on a literature review of theoretical co-culture studies, these studies tend to focus on aspects that are not easily investigated experimentally.

and extrinsic noise and simulate reactor capacities, seeding densities and seeding ratios that would support the proposed system. For this system, population trajectories become more variable in larger reactors, leading to a less predictable system. When population behaviours are controlled by inter-population metabolite exchange, population ratios, growth and death rates can determine culture fate as they determine concentrations of metabolites secreted by cells [52,62,100]. As substance exchange is controlled through intracellular genetic parts, well-characterized parts are required for predictable population behaviour.

For any co-culture model, it is important to consider whether the assumptions made match feasible experimental set-ups. Kambam *et al*. [101] simulated an artificial symbiosis between cell populations and show it to be stable in an open environment with continuous nutrient supply and that nutrient requirements determine strongly the possibility of stable long-term solutions. Therefore, possible experimental set-ups would need to reflect this. Mathematical modelling can replace large-scale and work-intensive experiments. Klitgord & Segrè [41] survey environments that induce synthetic microbial ecosystems by theoretically testing thousands of possible growth media, and the results suggest that the environment can have a greater effect on population interactions than cell genotypes. Therefore, efforts going into extensive genetic manipulation may be wasted if environmental set-up for testing is not taken into account.

Earlier studies showed how time-variant bioreactor conditions can allow stable co-existence of populations with different growth rates, by varying pH [43] or dilution rate [44,45], including coexistence of two populations of different species [43] or even three populations [45]. Modelling is here used to optimize the frequency of cycling conditions to obtain stable populations. Operating diagrams are used with great effect to illustrate areas in parameter space where species can coexist and what parameter combinations lead to other steady-state conditions such as death of all populations or survival of some. However, stabilizing populations through condition cycling may not be suitable for industrial or laboratory experiments, as changing conditions could introduce complex undesired dynamics.

Co-culture studies are complex and require extensive optimization. In one case using a three-step model, individual populations were first simulated in separate compartments, then in shared medium allowing each population to take up substances secreted by the other and finally in mixed culture [102]. A number of studies theoretically investigate existing experimental co-culture systems, showing that there is information to be extracted from these systems that cannot feasibly be done experimentally. Models relying on a large number of variables such as rate constants are suboptimal as this information may not currently be available and difficult to obtain experimentally and parameters obtained from *in vitro* monoculture are unlikely to be representative of the co-culture context [47]. As in synthetic biology in general, there is a need to integrate the modelling and experimental efforts, though currently lack of experimental data makes model design and validation difficult [99]. Discrepancy between modelling and experimental results may to some extent be caused by the specific experimental set-up, giving valuable insights into how the environment affects the system.

4. Available technologies for co-culture

For co-culturing populations with a degree of separation, commonly used options include transwell plates [56,103], microfluidic platforms [4,6,94,96] or solid supports such as Petri dishes [10,40], three-dimensional scaffolds [74], hydrogels [57] or microarrays [82]. Many bespoke microfluidic

devices have been designed specifically for co-cultures [11,12,22,36,53,58,62,73,76,79]. One of the main advantages of microfluidics is the potential for high throughput. However, with increasing complexity this advantage may be compromised [6] and microfluidic set-ups may not be representative of larger volumes. Monoculture techniques are also increasingly being adapted for co-culture, for example cell migration assays [79]. Conventional laboratory cultivation is mostly aimed at pure cultures of individual species [11,17]. The cultivation of monocultures on agar plates and in liquid medium has always followed the same paradigm [9,104], though there have been recent advances that could be further used, including microcarrier beads [5], the micro-Petri dish [104], diffusion chambers, dialysis reactors and other methods [9,17].

In synthetic biology, there is a drive towards techniques that are inexpensive and commonly accessible [67,81] (figure 3). On the other hand, there is also a need for high-content automated systems that allow for large amounts of data collection during co-culture. While characterization of monocultures is valuable for predicting co-culture interactions, such experiments cannot give complete information, meaning that co-culture characterization experiments are necessary. There are a number of capabilities that co-culture technology should provide. They should enable the study of several similar co-cultures (including test and control cultures) in parallel in the same device [23,36,79]. Technology should interface with existing imaging and robotics equipment [6]. The ability to install or remove a separation between populations would enable mixed and separated cultures to be grown under otherwise identical conditions, thus allowing the control of variables that have not previously been controlled. More generally, technology in synthetic biology should be modular to reflect the aims of the field and to make full use of the so-called 'plug and play' genetically engineered populations [26]. In the same way that simple transwell inserts transform culture plates into co-culture plates, attachable modules could be developed for many sizes and types of reactor. An existing example of this is a perfusion bioreactor system for mammalian cell co-cultures that can have modules added to it [70]. While engineered intercellular signalling may allow functional connection of an unlimited number of cell populations [26], the reactor to characterize such a system has yet to be developed. Isolated examples of co-culture set-ups were discussed here that provide for experiments requiring 'extremes' in terms of certain parameters like population number, volume or time scale, but advances in technology could make such experiments commonplace.

As co-culture experiments carried out in different kinds of vessel may not be comparable [10,39], it may be necessary to test systems across several platforms. This highlights the importance of documenting extensive metadata of co-cultures in publications, which is currently not always given, as this is invaluable for process replication and optimization. Studies should report on the variables mentioned herein: organisms cultured, technology, degree of separation, population densities and ratios, time scale and volume, as these have all been shown to influence co-culture fate. Overall, the desired working environment needs to be taken into account when designing synthetic gene circuits. On the other hand, improved availability of high-throughput co-culture testing methods would alleviate the need for extensive prior knowledge of gene circuit behaviour and allow wider use of synthetic biology approaches.

5. Data acquisition in co-cultures: further challenges

Detailed data acquisition for co-cultures presents a great challenge. Co-cultures are carried out for a number of reasons, which define the parameters to be monitored. A major aim of laboratory-based co-cultures tends to be characterization of system behaviour. Industrial scale-up, medical and environmental applications require in-depth data collection and characterization [96] and the co-culture set-up should be carefully considered beforehand to allow for the required data collection. This includes consideration at the stage of genetic circuit design to possibly include fluorescent output in response to relevant inputs. Not all methods of data acquisition may be compatible with eventual industrial, medical or environmental applications. Therefore, consideration should be given early in the development process to how co-culture behaviour will be monitored at the later stages of process development.

Data acquisition must be carefully planned. There is generally a need to characterize the activities and dynamics of each cell population in detail, as well as the interpopulation interactions and growth medium composition. Any information about the populations in a co-culture can potentially be used to control cell behaviour [16]. Tools for characterization and quantification of mixed microbial cultures have previously been discussed [17,105] and include largely the same methods as used in monocultures. Flux between cells is more easily measured than flux within cells, giving co-cultures an advantage over monocultures for obtaining insights into metabolism [29]. However, meaningfully measuring the biologically relevant interactions between populations can be difficult [17,64], because populations may exchange more than is known, even in synthetic communities [99]. Studying interactions remains a major challenge and controlling them even more so [17].

For monocultures, there is a drive towards equipment allowing online data collection, such as simple plate readers or more complex systems such as the BioLector (m2p labs). However, such systems may not always be suitable for online monitoring of multiple populations in a co-culture, as optical density cannot be used to measure population density in co-cultures [99] and co-culture equipment such as transwells may not easily interface with plate readers. Therefore, most studies using co-cultures perform either end-point data collection or sampling during the course of the culture for off-line analysis.

Full characterization of co-culture dynamics probably requires a whole series of experiments starting with characterization of isolated parts, followed by each population in isolation and leading to full co-culture of several populations under a number of conditions and including mathematical modelling at every stage. Payne *et al*. [91] provide detailed and specific protocols for quantitative analysis of the synthetic predator–prey system previously presented by Balagaddé *et al*. [62] in Petri dishes and culture tubes. Kato *et al*. [55] present 'knockout cultures' to understand the role of each population in a cellulose-degrading community of five bacterial strains. In subsequent work, one of the stable

knockout co-cultures (made up of four populations) is analysed in detail for interpopulation interactions. All population combinations are cultivated and populations are exposed to spent medium from other populations [106]. However, while these experiments give a great amount of insight, they cannot fully define the interactions in the final co-culture [17]. Several recent studies have presented complete experimental and theoretical strategies for co-culture characterization [98,99].

6. Conclusion and future perspectives

Synthetic biology aims to genetically engineer biological systems for complex applications. Many of these systems involve multiple cell populations. Out of the 20 most highly cited primary articles on Web of Knowledge that contain the term synthetic biology, at least a quarter contain a form of co-culture. Co-cultures are said to potentially present a whole range of desirable characteristics over monocultures: modularity, robustness, predictability, scalability and stability [29]. Already, bacteria engineered for tumour targeting and therapeutic delivery are being tested in animal models [107] and clinical trials [37]. However, these are isolated successes, as such systems require extensive *in vitro* characterization in terms of function and safety before being used. The aforementioned desirable characteristics can only be achieved through extensive experimental and theoretical testing and characterization of population behaviours. Such characterization is challenging, as synthetic multi-population systems are some of the most complex systems described to date and currently the best characterization data in synthetic biology is at the level of devices [39].

The efforts in synthetic biology towards engineering tools for co-cultures are largely targeted at the genetic engineering of populations rather than the cultivation protocols and technologies. A complete design cycle, however, includes extensive testing and characterization, therefore co-culture systems are essential for the synthetic biology vision of engineering biology. Even cultivation of single species is not straightforward because of the development of heterogeneous phenotypes and asynchronous behaviour [63]. Furthermore, the full effects of different common cultivation methods on cell phenotype are still unknown. Cultivation of several populations is much more complex yet, as co-culture behaviour is more than the sum of the individual population activities. The problem is compounded by the fact that there is a lack of fast, simple, inexpensive assays for co-cultures to enable such experiments to be carried out more routinely for different conditions and species, at low cost, without the requirement for extensive technical expertise [99]. While there are extensive ongoing efforts to automate and optimize the processes at every step of the synthetic biology pipeline, e.g. gene circuit design and assembly and characterization of isolated parts, there has been a comparative lack of advances in cultivation methods compared with other parts of the pipeline [9].

Overall, the aim is characterization of populations and their interactions to the same level as currently possible for parts and devices including extensive documentation of experimental conditions. As population interactions suffer from context dependency and can change in different environments, characterization should include stress limits. For example, an engineered interaction was reported to be functional only within certain pH ranges [39]. Taking context dependency into account does not imply a departure from the engineering and computing analogy on which synthetic biology is based, but rather an improved appreciation of the challenges that come with the analogy. Just as civil engineers need to consider the environmental stresses that a building will have to withstand, synthetic biologists need to consider the working environment of bio-parts at the stage of circuit design.

On the other hand, co-culture technologies can support the genetic engineering that is at the basis of synthetic systems. Genetic programming cannot entirely predict cellular behaviour, as environment plays a role and there are still limits in what can be controlled genetically [41]. Using genetic engineering and controlled environmental conditions through experimental set-up together could allow a level of population control that was previously thought impossible. Emergent and unpredicted behaviour can arise in co-cultures and may or may not be desired. One important question in the choice of cultivation technology is what properties of the natural populations are to be preserved or suppressed. Overall, populations can be controlled genetically and through the environment. Which aspects can be controlled genetically or through set-up is a subject of ongoing research.

Co-culture technology represents the link between genetic engineering and application of complex systems. Co-cultures can cover many levels of the biological hierarchy like organs and ecosystems, meaning that technology development investment will enhance research in more than one area [2]. Availability of co-culture technology could allow answering a huge range of biological questions. It has been stated that industrial microbes should display robustness, stability of metabolism in changing environments, tolerance to toxic metabolic waste or compounds in feedstock and resistance to environmental stresses, characteristics that are not easily engineered in a single microbe [21,108], but may be fulfilled using consortia if suitable enabling technology is available.

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