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## Self-organization of *Saccharomyces cerevisiae* colonies

Zoran Marinkovic

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Thèse de doctorat

de l'Université Sorbonne Paris Cité

Préparée à l'Université Paris Diderot

**Ecole Doctorale Interdisciplinaire Européenne Frontières du Vivant (ED 474)**

*Laboratoire Matière et Systèmes Complexes (MSC)*

*et*

*INSERM U1001*

# **Self-organization of *Saccharomyces cerevisiae* colonies**

Présentée par

**Zoran MARINKOVIĆ**

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Thèse de doctorat de biophysique

Dirigée par Pascal HERSEN et Ariel B. LINDNER

Présentée et soutenue publiquement à Paris le 30 novembre 2017

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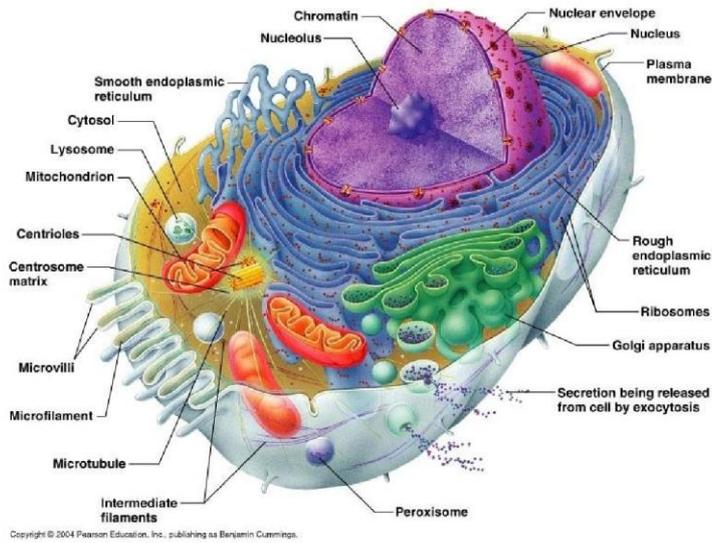


*To the rare reader of this manuscript:*

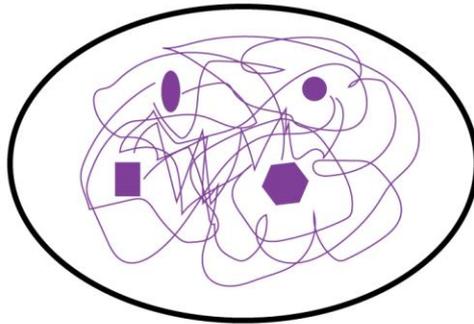
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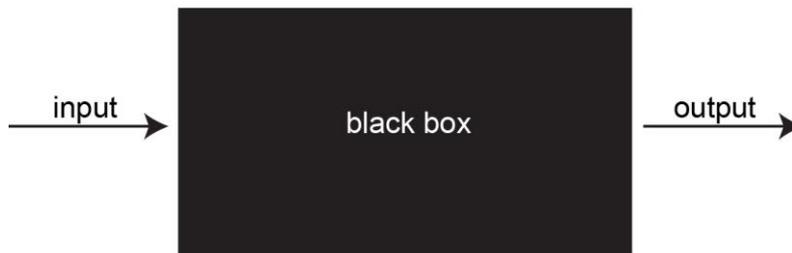
# The view of a cell by:



a biologist



a chemist



an engineer



a physicist

Figure 1 (previous page) This figure was inspired by an internet meme. *“Examples of memes are tunes, ideas, catch-phrases, clothes fashions, ways of making pots or of building arches. Just as genes propagate themselves in the gene pool by leaping from body to body via sperms or eggs, so memes propagate themselves in the meme pool by leaping from brain to brain via a process which, in the broad sense, can be called imitation.”* – Richard Dawkins, “The selfish gene”, 1976;

*“[In internet memes] mutations are designed, not random, with the full knowledge of the person doing the mutating”* – Richard Dawkins, Saatchi & Saatchi New Directors' Showcase, Cannes, 2013;

<https://www.youtube.com/watch?v=2tlwYNioDL8> – Richard Dawkins – Memes vs Genes song

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# Abstract

The natural environment of yeast is often a community of cells but researchers prefer to study them in simpler homogeneous environments like single cell or bulk liquid cultures, losing insight into complex spatiotemporal growth, differentiation and self-organization and how those features are intertwined and shaped through evolution and ecology. I developed a multi-layered microfluidic device that allows us to grow yeast colonies in spatially controlled dynamically structured changing environments from a monolayer of single yeast cells to a multi-layered colony. Colony growth, as a whole and at specific locations, is a result of the nutrient gradient formation within a colony through interplay of nutrient diffusion rates, nutrient uptake rates by the cells and starting nutrient concentrations. Once a limiting nutrient (e.g. glucose or amino acids) is depleted at a specific distance from the nutrients source the cells within a colony stop to grow. I was able to modulate this specific distance by changing the starting nutrient concentrations and uptake rates of cells. Colony gene expression patterns gave us information on specific micro environments formation and consequential development, differentiation and self-organization. I quantified the patterns of expression of seven glucose transporter genes (HXT1-7), each of them specifically expressed depending on the glucose concentration. This enabled us to reconstruct glucose gradients formation in a colony. I further followed the expression of fermentation and respiration specific genes and observed differentiation between two subpopulations. We also mapped other genes specific for different parts of carbohydrate metabolism, followed and quantified the spatiotemporal dynamics of growth and gene expression, and finally modelled the colony growth and nutrient gradient formation. For the first time, we were able to observe growth, differentiation and self-organization of *S. cerevisiae* colony with such an unprecedented spatiotemporal resolution.

## Résumé

L'environnement naturel des levures est constitué d'une communauté de cellules. Les chercheurs, cependant, préfèrent étudier les levures dans des environnements plus simples et homogènes, comme des cultures en cellule unique ou en population, s'affranchissant ainsi de la complexité de la croissance spatiotemporelle, la différenciation, l'auto-organisation, ainsi que la façon dont ces caractéristiques sont formées et s'entrelacent à travers l'évolution et l'écologie. Nous avons mis en place un dispositif microfluidique multicouches permettant la croissance de colonie de levures dans des environnements dynamiques, spatialement structurés, contrôlés, partant d'une monocouche de levures à une colonie multicouches. La croissance des colonies, dans son ensemble comme à des positions spécifiques, est le résultat de la formation d'un gradient de nutriment au sein de celles-ci - gradient qui trouve son origine dans le différent taux de diffusion des nutriments, des taux d'absorption de ceux-ci par les cellules, ainsi que de leurs concentrations initiales. Lorsqu'un nutriment en quantité limitante (par exemple le glucose ou un acide aminé) est épuisé, à une distance spécifique de la source de nutriments, les cellules au sein de la colonie cessent de croître. Nous avons été en mesure de moduler cette distance spécifique en variant la concentration initiale de nutriments ainsi que le taux d'absorption des cellules. Les motifs d'expression de gènes de la colonie nous ont donné des informations sur la formation de micro environnements spécifiques ainsi que sur le développement subséquent, la différenciation et l'auto-organisation. Nous avons quantifié les motifs d'expression de sept gènes de transport du glucose (HXT1-7), chacun exprimé spécifiquement suivant la concentration de glucose, ce qui nous a permis de reconstituer la formation de gradients de glucose au sein d'une colonie. En étudiant des gènes spécifiques de la fermentation et de la respiration, nous avons pu observer la différenciation en deux sous-populations. Nous avons de plus cartographié l'expression de gènes impliqués dans différentes parties du métabolisme des glucides, suivi et quantifié la dynamique spatio-temporelle de croissance et d'expression génétique et finalement modélisé la croissance de la colonie ainsi que la formation du gradient de nutriment. Pour la première fois, nous avons observé la croissance, la différenciation et l'auto-organisation des colonies de *S. cerevisiae* avec une résolution spatio-temporelle jusqu'à maintenant inégalée.





# Foreword

What happens when one - an atom, a molecule, a cell, or an organism - finds itself in a group of more than one, is the core question of this thesis. I am using a well-established model organism, yeast *Saccharomyces cerevisiae*, to study its growth, differentiation, and self-organization when single cells come together and grow as a colony. Ever more often, the call to put microbiology research in a more natural context can be encountered in papers. We have benefited greatly from Cartesian reductionism, or even better - methodological reductionism, where we would observe and measure parts of a system isolated from a system, like decoding the gene function of a single gene, taking proteins out of cells and crystalizing them to deduce their function from their structure, plucking out bacteria or yeast cells from their environment and growing them in a well-mixed homogeneous liquid culture which they hardly encounter in nature, or just the notion of a model organism itself where one species of bacteria or one species of yeast should represent an estimated one trillion species of microbes on Earth. Does a fashion supermodel represent an average human being? In some aspects, hardly. Reductionism works because systems are indeed made of constitutive parts which have their own observable and measurable properties or, in biology, evolutionary conservation is undeniable and it is true that we can learn much about the principles of life from a model organism. However, this is not a complete picture. Parts of a system can exhibit new, on an individual level non-existent, emerging properties when they come together and form a system. The whole is greater than the sum of its parts, it is often said, and we are becoming increasingly aware how complex and intertwined biological systems are. Even the simplest ones as bacteria or yeast. As a physicist and a Nobel laureate Philip W Anderson has put it in 1972 in his widely cited essay *"More is different"* [1]: *"At each stage [of organization] entirely new laws, concepts, and generalizations are necessary... The arrogance of the particle physicist and his intensive research may be behind us (the discoverer of the positron said "the rest is chemistry"), but we have yet to recover from that of some molecular biologists, who seem determined to try to reduce everything about the human organism to "only" chemistry, from the common cold and all mental disease to the religious instinct."* It is similar with interdisciplinarity. While it is useful to systematize knowledge into boxes like physics, chemistry, cell, molecular, evolutionary biology, or ecology, it might be perilous to gather knowledge in such an entrenched way as many problems need complex approaches that combine methods and knowledge of many of these fields. Nature does not know or care about them anyway.

Personally, I am a molecular biologist, gene systems and synthetic biologist, gene biophysicist with some aspects of ecology, but most importantly I am a scientist. And I think that reductionist and systems approaches are of complementary nature, one can inform the other and vice versa.

This is why my work is somewhere in between these two worlds. We use an established model system for simplicity, genetic tractability, and extensive knowledge of its function, but we listen to the calls for the need of bringing research closer to the phenomena occurring in nature. In nature, microorganisms live in dynamic environments and they often form communities of cells. When single cells grow together they uptake nutrients from the environment and they release molecules into the environment, effectively constantly changing their environment. The environment has an effect on cells as they adjust their cellular response to the environment, but they also form their environment in a constant feedback loop. This heterogeneity of the environment is often omitted in the laboratory conditions as researchers simplify the relationship between the cells by making the environment homogeneous. We devised a method, based on the principles of microfabrication and microfluidics, where we can grow a monolayer of yeast cells into a colony of large enough dimensions that cells “self-inflict” nutrient gradients upon them through the interplay of diffusion and nutrient uptake by cells. This forms local microenvironments and landscapes of growth and gene expression differentiation within a colony, where different populations show different phenotypic properties depending on their position in the colony. This emerging phenomenon would not be possible to observe in single cells, but rather it happens only when cells grow together and form a new level of organization where they interact through, what we call, long-range metabolic interactions within a colony. We believe that this is a step closer to quantification of biological and physical phenomena that happen in nature, where cells often grow together in formations of many cells, but still it has the simplicity and tractability for use in the setting of a research laboratory.

# Thesis organization

I will first give an introduction on the problematics of self-organization of single cells into colonies and establish a connection with the evolution, in particular the emergence of sociality and multicellular organisms on Earth. Next, I will explain why is yeast *Saccharomyces cerevisiae* such an important model organism and where can we find it in nature. We will then submerge inside yeast and see how its metabolism works, and what are the most important mechanisms in nutrient sensing, signalling, and transport. After that we will see what kind of implications this has on growth of yeast in homogeneous liquid cultures and in colonies, together with a review of some growth models. I will then present the microfluidic device that we have built and describe the results that we managed to obtain. I will first focus on the quantification of growth of yeast colonies both as a single entity and at local positions inside it, across a wide range of starting glucose concentrations. Then, I will present the gene expression landscape within colony of key glucose transporters and a method we devised to reconstruct the glucose concentration gradient that is formed within a colony. Next, we will look at other glucose related phenomena that happen within a colony, like growth arrest, differentiation between populations that ferment and respire, glucose repression, and glycolysis. I will conclude on the dependence of growth and gene expression in changing glucose concentrations, and switch to their dependence on amino acid concentrations within a colony. Finally, we will see what happens when we delete glucose transporters and glucose sensors from the yeast. At the very end, there is a brief overview of the evolution of the design of our microfluidic device after which I very briefly present other side projects that I worked on during my PhD.



# Introduction

## Self-organization of single cells into colonies

Living in a community is an emergent property of life — interactions between individuals and their environment give rise to complex behaviours. Populations of microorganisms like bacteria *Escherichia coli* or yeast *Saccharomyces cerevisiae* are not simply an independent sum of all individuals that make them, but rather it is a complex structure with many intricate interactions which produce coupled spatiotemporal variability. It is an interdependent community of many cells composed of different local microenvironments with specific growth rates as well as production and uptake of nutrients, wastes and other molecules. There are many environmental and genetic determinants that give rise to a complex colony morphology and internal dynamics, such as intercellular communication, cell surface properties, cell-cell adhesion strength, sensing nutrients level, emergence of local microenvironments, and secretion of extracellular matrix [2]. Such communities composed of many cells exhibit various adaptive benefits like more efficient proliferation, access to resources and niches that require a critical mass and cannot effectively be utilized by isolated single cells, collective defense against antagonists that usually eliminate isolated single cells, and optimization of population survival when confronted with diverse physical, chemical, nutritional or biological challenges [3]. In spite of the obvious contrast between homogeneous environments on one side and heterogeneous environments on the other, the majority of scientific research until now has been focused either on single cells, continuous, and bulk liquid cultures, putting aside the complexity of communities composed of many cells – the most encountered microorganism formation in nature, which can consist of near clonal individuals, genetically distinct individuals of the same species, mixed populations of different species, or microbial-host interactions (Figure 2).

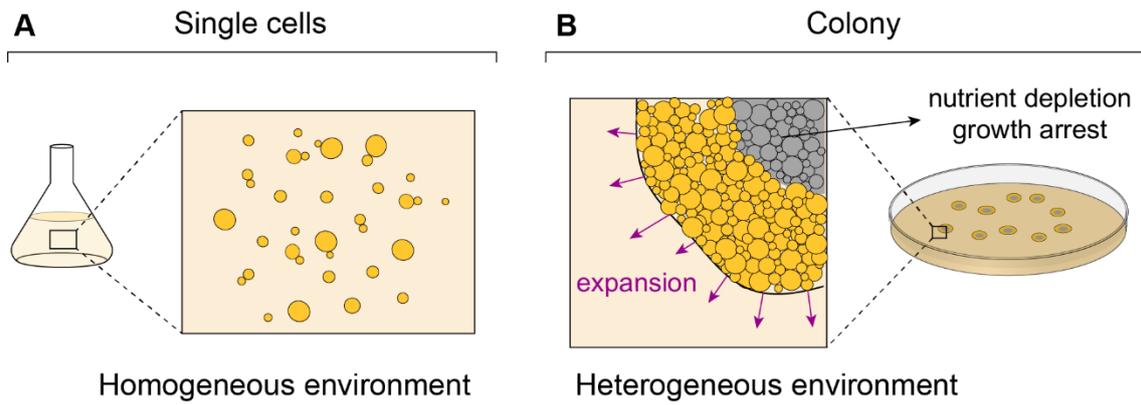


Figure 2 Two distinct types of cell population structure. (A) Single cells in a homogeneous environment, either well mixed or constantly replenished, are considered to have similar properties and clear comparable relationships between each other (e.g. growth rates). (B) Cells living as a colony form heterogeneous environments within a colony and show distinct properties depending on their position in the cell assembly.

This simplification of thinking about single cell microorganisms by considering them individually is not surprising as it has yielded significant advances in understanding the principles of life [4]. However, with the advances in technologies like microfluidics, genomics, transcriptomics, proteomics, metabolomics, interactomics, metagenomics, genetic engineering, robotics, modeling and simulation, we are able to gain insight with high efficiency and throughput, as never before, in the way cells function. From precise molecular details of a single cell to controlling structure, interactions and dynamic environments of cell populations, even mimicking the function of different organs. With this, we can embark on a journey to explore topics of higher complexity, integrating different methods and insights gained through the reduction of complexity into a systems-level understanding (Figure 3).

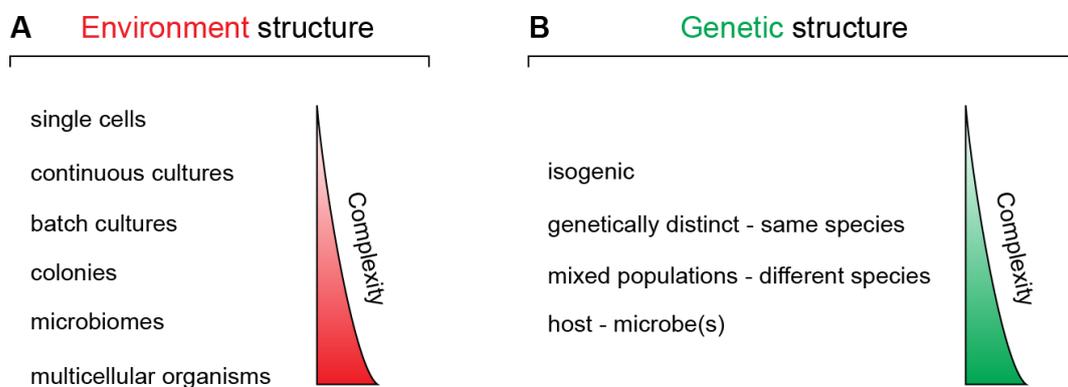


Figure 3 Rough distinction between the level and the type of complexity. Scientists like to simplify their research so most of the studies in microbiology are done on isogenic single cells or populations in homogeneous environments in continuous or batch cell cultures.

The simplest case in terms of the structure of the environment and genetic diversity is the case where isogenic single cells are observed with media constantly replenished. An example of this would be single cells separated from each other in a microfluidic chip under constant flow of nutrients. In this case cell response is just the product of the environment that we decided to impose on cells. In continuous culture, like chemostats, media is constantly replenished and the culture is mixed so environment is homogeneous but nevertheless cells do release metabolites in the environment so at least in a small part they do slightly change the environment that we want to impose on them. In batch culture, culture is mixed so the environment is homogeneous, but media is not replenished, hence cells consume nutrients and release metabolites into the environment, directly changing the environment which eventually leads to the exhaustion of the media, growth arrest and death. In colonies (or any other similar structure, like biofilms), nutrients diffuse into the colony and cells uptake nutrients, which forms gradients of nutrients and local microenvironments. Cells also produce metabolites that are released into their immediate environment which adds to the colony complexity. In all of these cases we can also introduce genetic structure, meaning that we can look at isogenic cells, same species but with some genetic distinction or populations of multiple species. On top of that, there are even more complex structures like microbiomes, host to microbe(s) interactions, and “true” multicellular organisms of different levels of complexities. It needs to be noted that this is a rough distinction and that some elements might overlap or contain variable levels of complexity within themselves.

## **Emergence of multicellularity**

Thinking about self-organization of single cells into assemblies of many cells inevitably leads to the reflexion on unicellularity, multicellularity, and individuality. Once upon a time, there was a popular theory which stated that all organisms were created at the same time and existed ever since. The most visually remarkable interpretation depicted a small human inside a sperm just waiting to grow in size. In every step on the way in description what life is, we were accompanied by technology. It was the discovery of the microscope which led the way to the discovery of plant and animal cells, as well as microorganisms or “animalcules” (“little animals”) as van Leeuwenhoek called them in the 17<sup>th</sup> century. Already then, he correctly estimated that there were more “animalcules” swimming and creeping around in his mouth than there were people living in the Netherlands [5]. Gilbert,

Sapp, and Tauber claim that we perceive only that part of nature that our technologies permit, and that our theories about nature are highly constrained by what our technologies enable us to observe [6]. Current technologies have shown us a microbial world of higher diversity than we thought before, with complex and interconnected relationships between microbes themselves, but also between microscopic and macroscopic life [7]. Gilbert *et al.* take the position of a symbiotic way of life, claiming that the contemporary discoveries profoundly challenged the generally accepted view of “individuals” and that symbiosis is becoming a core principle of biology, *“replacing an essentialist conception of “individuality” with a conception congruent with the larger systems approach now pushing the life sciences in diverse directions... that transcend the self/nonsel, subject/object dichotomies that have characterized Western thought”*. This idea was fuelled with the magnitude of interactions we discover every day between humans and their microbiome, which even includes some evidence that this interaction can affect our behaviour and perhaps increase the chance of some neuropsychiatric illnesses [8], [9]. There are even bacteria inside our tumours, thriving in the niche of the intratumor microenvironment, that can mediate tumour resistance to anticancer drugs [10].

This is not a thesis in experimental evolution so I will not dwell further on the feud between neo-Darwinism and symbiogenesis [11], [12], and I will leave it at a short exchange between the two “controversial”, flamboyant, and highly influential evolutionary theorists, which happened in 2009 during “*Homage to Darwinism*” debate held at Oxford University:

Richard Dawkins: *“If you take the standard story for ordinary animals ...what’s wrong with it? You’ve got a distribution of animals, you’ve got a promontory or an island ...so you end up with two distributions... And then on either side of this promontory you get different selection pressures, so this one starts to evolve that way, this one starts to evolve that way, and what’s wrong with that? It’s highly plausible, it’s economical, it’s parsimonious. Why on Earth would you want to drag in symbiogenesis when it’s so unparsimonious and uneconomical?”*

Lynn Margulis: *“Because it’s there.”*

Indeed, interactions between different constituents of the environment are all around us and we need to take them into account when we think about evolution, ecology, and emergence of different cellular functions. No matter what we think about the questions like “Is cow a cow without its cellulose-digesting bacteria?” [13], “Is human a human without its 38 trillion bacteria?” [14], or “Is coffee a coffee without its caffeine?”, the fact is that the environment is full of proliferating entities which are seldom found as a single unit, but rather they often form aggregates or communities of many units, even if it is only during division. They can be composed of unicellular and/or multicellular species which will be the product of their environment, but also their environment will be the product of them, most likely forming heterogeneous environments in which each cell will exhibit a phenotype specific for such an environment based on its (epi)genetic program and current phenotypic state, which evolved to a different degree of complexity as a consequence of previous history of adaptation, chance, interaction, and in the contemporary time of synthetic biology, “rational” design by humans. It is ok if we observe parts of a greater assembly isolated from such an assembly, but we need to be aware that we lose the complete picture and that we are discovering only a partial phenomenon, because as Aristotle has put it *“The whole is greater than the sum of its parts”* or in a longer form in a proper context from his *“Metaphysics”*: *“To return to the difficulty which has been stated with respect both to definitions and to numbers, what is the cause of their unity? In the case of all things which have several parts and in which the totality is not, as it were, a mere heap, but the whole is something beside the parts, there is a cause; for even in bodies contact is the cause of unity in some cases, and in others viscosity or some other such quality. And a definition is a set of words which is one not by being connected together, like the Iliad, but by dealing with one object. What then, is it that makes man one; why is he one and not many, e.g. animal + biped, especially if there are, as some say, an animal-itself and a biped-itself? Why are not those Forms themselves the man, so that men would exist by participation not in man, nor in-one Form, but in two, animal and biped, and in general man would be not one but more than one thing, animal and biped?”* One also needs to wonder why everyone is so anthropocentric when there are one million trillion trillion microorganisms out there [15].

From an evolutionary point of view, there were several major transitions that happened during evolution of life, which by and large include [16]:

- I. the compartmentalization of replicating molecules, yielding the first cells
- II. the coalescence of replicating molecules to form chromosomes
- III. the use of DNA and proteins as the fundamental elements of the genetic code and replication
- IV. the consolidation of symbiotic cells to generate the first eukaryotic cells containing chloroplasts and mitochondria
- V. sexual reproduction involving the production (by meiosis) and fusion of haploid gametes
- VI. the evolution of multicellular organisms from unicellular ancestors
- VII. the establishment of social groups composed of discrete multicellular individuals

Multicellularity independently evolved at least 25 times from unicellular ancestors (Figure 4) [16]. It evolved in prokaryotes, as well as in eukaryotes. Even today multicellular organisms emerge from unicellular predecessors [17], but they can also revert to unicellular state, as in the example of bacteria *Myxococcus xanthus* which was grown for 1000 generations in liquid culture at low densities (n.b. in lab conditions, hijacked from its natural environment!) and consequently displayed significantly reduced social behaviour, which was dependent on cell-cell interactions and extracellular matrix production [18]. The notions of multicellularity can be blurred. In slime molds, unicellular and multicellular states alternate depending on the environmental cues [19], [20]. Slime mold *Dictyostelium discoideum*, upon starvation, forms a fruiting body which consists of dead stalk cells which promote the dispersion of the dormant spores (that can stay alive for months in starvation) towards nutrient-rich areas (Figure 5 B). However, in response to the fluctuating environment different strategies may emerge. As Dubravcic, van Baalen, and Nizak have recently discovered, up to 15% of single cells of *Dictyostelium discoideum* do not aggregate to form the multicellular fruiting body, as non-aggregating cells have an advantage over cells in aggregates because they can resume growth earlier upon arrival of new nutrients at the expense of a shorter lifespan under prolonged starvation [21]. In case of bacteria and yeast the theoretical ponderings about what constitutes multicellularity is even more vague. Although they are mostly unicellular (there are some cases of multicellularity in

bacteria), they can form complex and organized communities, like biofilms, colonies, flocks, and mats, which might exhibit some prerequisites of multicellularity (Figure 5 C). For example, in terms of differentiation we have shown that cells can differentiate within a colony. But this differentiation probably emerges as a consequence of local microenvironment which is formed by the emergent self-organization and collective behaviour of cells that make a colony (and vice versa, cell-environment interactions go both way). In this case we might need to make a distinction between “phenotypic differentiation” and “programmed differentiation” [13]. It implies some kind of “pre-built” intention or stable “specialization” recognized through potential benefits that we might measure, which is not that completely clear in the cases when bacteria or yeast grow together and form assemblies of single cells.

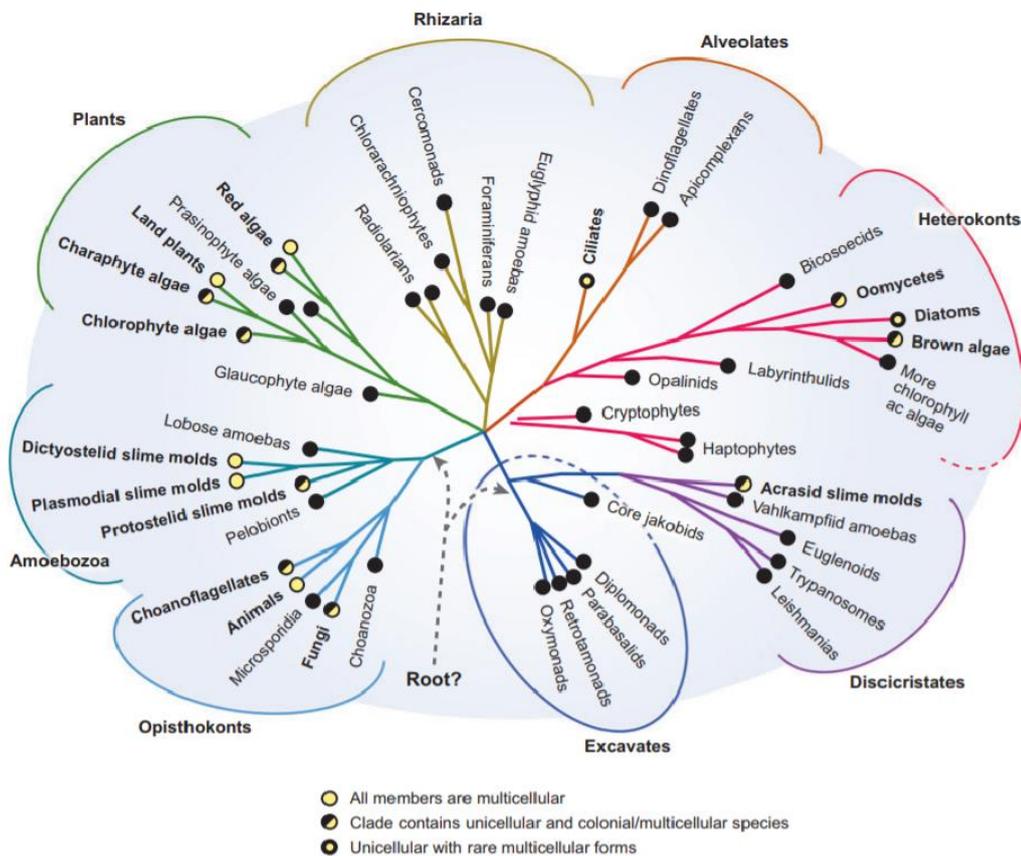


Figure 4 The phylogenetic distribution of multicellularity among eukaryotes. Adapted from [16].

In 1998 Shapiro published paper “*Thinking about bacterial populations as multicellular organisms*” in which he argued that we should consider bacteria as multicellular organisms [3]. He summarized his core concepts into following points:

- I. Bacterial cells have communication and decision-making capabilities that enable them to coordinate growth, movement, and biochemical activities
- II. Examples of communication and coordinated behaviours are widespread (possibly ubiquitous) among bacterial taxa and are not limited to a few groups with a specialized multicellular vocation
- III. Bacterial populations derive adaptive benefits from multicellular cooperation and their ability to integrate the diverse activities of different cells. These benefits include (but are not limited to):
  - a) More efficient proliferation resulting from a cellular division of labour
  - b) Access to resources and niches that cannot be utilized by isolated cells
  - c) Collective defence against antagonists that eliminate isolated cells
  - d) Optimization of population survival by differentiation into distinct cell types

However, in 2008 Nadell, Xavier, and Foster have challenged this view in their paper “*The sociobiology of biofilms*” [22]. They claim that the uncoordinated behaviour of many cells responding to heterogeneous microenvironments may give rise to complex biofilm structures (e.g. phenotypic differentiation, species stratification, and channel formation) and that this observation suggests that any explanation of biofilm structure that assumes whole-biofilm coordination must be tested against the more parsimonious hypothesis that biofilm structure does not strictly depend on intercellular communication. They further conclude that the evolution of cooperation among all cells is unlikely for most biofilms, although local cooperation among bacteria may often occur.

Sometimes the living world is divided into unicellular, colonial, and multicellular organisms, where colonials are unicellulars living together [23]. This is for sure a convenient separation but it might omit the complexity as previously discussed. No matter how we classify organisms, it is important that there is a univocal agreement that social interactions of different complexities do happen at every level in the ecosystem. Finally, even though multicellular aspects of

assemblies of unicellular organisms is a debatable topic, in many cases multicellularity is clear, even on the intuitive level. For example, in the cute *Anabaena*, a cyanobacteria that can both do photosynthesis and nitrogen fixation (Figure 5 A). This process could not happen in a single cell because oxygen produced during photosynthesis inactivates nitrogenase required for nitrogen fixation [24]. *Anabaena* can produce a specialized cell called heterocyst that lacks chlorophyll but synthesizes nitrogenase which converts nitrogen gas into ammonia or nitrate. This heterocyst is easily distinguishable by eye as it appears every ten to twenty cells in a filament of otherwise identical cells and it is essential for survival as it fixes nitrogen for amino acid production and cellular biosynthesis. The other intuitive example of a multicellular organism would be, well, a human being. As a nice review on the topic of unicellularity/multicellularity and the requirements to describe an organism multicellular (e.g. cell adhesion, cell-cell communication and coordination, programmed cell death (PCD), epigenetic modification of patterns of gene expression, division of labour), the reader should refer to Grosberg's and Strathmann's "*The Evolution of Multicellularity: A Minor Major Transition?*" [16].

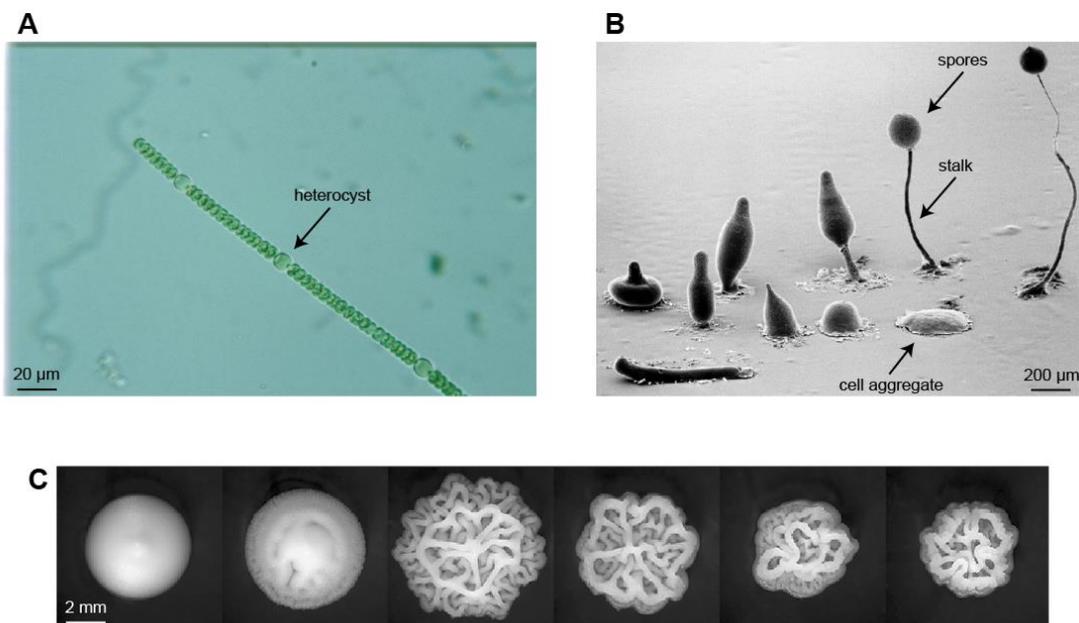


Figure 5 Different types of microorganisms. (A) Cyanobacteria *Anabaena torulosa*. Nitrogen fixing heterocyst is easily distinguishable by eye as it appears every ten to twenty cells. Adapted from protist.i.hosei.ac.jp. (B) Slime mold *Dictyostelium discoideum*. ~24h lifecycle, from cell aggregate to full fruiting body with stalk and spores. Adapted from dictybase.org. Copyright, M.J. Grimson & R.L. Blanton Biological Sciences Electron Microscopy Laboratory, Texas Tech University. (C) Yeast *Saccharomyces cerevisiae*. 5 day old colonies in different glucose concentrations (from left to right 2% to 1/16% in two-fold steps) Adapted from [2].

Since multicellular organisms are descendants of single cell organisms, so it comes as no surprise that researchers use bacteria or yeast to study possible paths to multicellularity using experimental evolution. Microorganisms are a very handy tool in the study of evolution and other branches of biology. They have short generation times, we can preserve them by freezing and then unfreeze them for use in the experiments, they are easy to grow in laboratory conditions, and we developed good molecular biology tools through decades of research. Both organisms have been suggested and used as good model organisms for elucidating the emergence of multicellularity [3], [25]–[28]. I will present several experiments with bacteria and yeast where the least common denominator is the ability to produce some kind of aggregation, post-division adhesion, clumping, or simply living tightly together, which makes sense if multicellular organisms arose from single cell organisms, they had to somehow stick together.

One of the most important prerequisite for emergence of multicellularity is cooperation between cells [29]. But usually this comes at a cost, cooperation can lower the fitness of co-operators and give rise to cheaters [30]–[32]. Cheaters are cells which benefit from the co-operators but they do not contribute to the benefit of the community. Typical example is excretion of some public good, a molecule all cells can use. Since production of such a molecule is costly for producer cell, a cheater, which does not produce this molecule but can benefit from it, has a fitness advantage and eventually it may take over the population. This leads to a conflict which needs to be resolved in order to evolve a multicellular organism, so it was proposed that in order to transition to multicellularity, cheating needed to be combated or suppressed [33]. However, Rainey and his colleagues took a different approach. They inversed the problem and asked themselves if this conflict could be used as a fuel for evolution, such that, in a way, cheaters become a germ line, a mean of reproduction of multicellular formation. Indeed, during more than a decade of work on aerobic bacteria *Pseudomonas fluorescens* they showed that such an evolutionary scenario might have played out [25]. The scenario goes like this (Figure 6): First, bacteria is inoculated in the media in the flask. Cells grow at the surface and stick to each other because they produce and adhesive substance. Eventually, they cover the whole surface, they form a mat closest to the source of oxygen which they need to survive. Due to mutation, a cheater which does not produce the adhesive substance arises, which makes the cheater more fit and in turn it grows faster than other cells. When the mat becomes too heavy due to cheater (and co-operator) propagation, it collapses, but the cheater can survive because it does not adhere to the mat. A new back-mutation arises and the progeny of the cheater become

adhesive co-operators again, so the life cycles completes. In this case cheaters are the propagating units (“germ line”) which arise from the mat (“soma”).

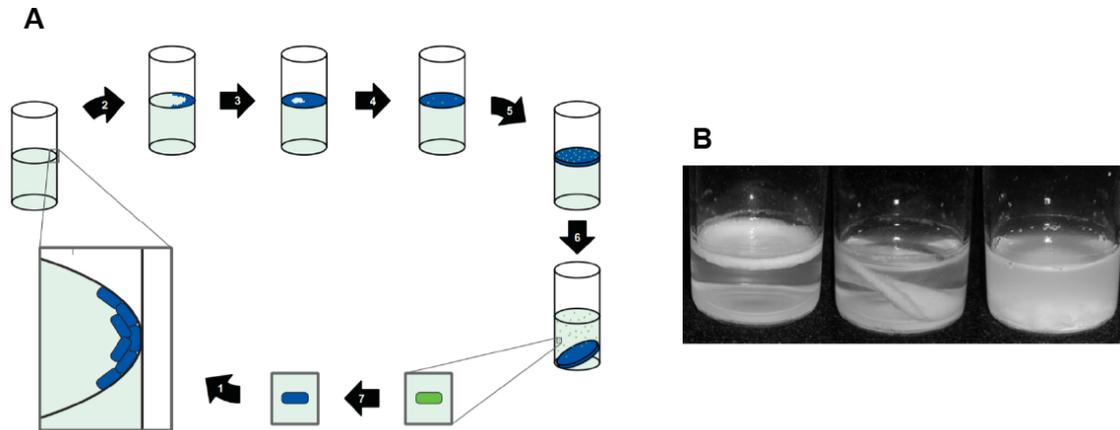


Figure 6 Aerobic bacteria *Pseudomonas fluorescens* as a model for the emergence of multicellularity. (A) Proposed life cycle for the mat-forming bacteria (explained in the text). (B) Emergence and the collapse of the mat composed of millions of bacteria. Figure adapted from [34].

Murray and his colleagues focused on the yeast *Saccharomyces cerevisiae* [35], [36]. This yeast produces an enzyme called invertase (SUC2 gene) which turns disaccharide sucrose into monosaccharides glucose and fructose. Sucrose cannot be imported by yeast cells, so invertase is expressed at the membrane where it hydrolyses sucrose and subsequently glucose and fructose can be imported in the cell via membrane transporters. Single cells will have a hard time using produced glucose and sucrose because it diffuses into the environment (Figure 7 A). However, if cells stick together, the local concentration of glucose and fructose will increase and cells will be able to use them more efficiently. This is indeed what happened. Cells that clumped together due to incomplete mother-daughter cell separation propagated faster than single cells (Figure 7 B). This is one scenario how multicellularity might have emerged without the need of complex communication or specific cell differentiation. In the follow up experiment they combined rational design (engineered mutation) with experimental evolution (allowing colonies to grow under selection pressure and studying the strategies that they adopt) [36]. They designed three yeast strains: one that formed multicellular clumps, the other that could import sucrose before hydrolysis, and the third with increased invertase expression. Using all three strategies helped yeast to grow in low sucrose. But then they wanted to check if these traits could evolve spontaneously? They tested this by

introducing wild-type yeast cells into a low-sucrose environment. They found 12 populations that survived: 11 evolved multicellular clumps and 10 increased the invertase expression. None of them evolved the ability to import sucrose. They also noticed that cells evolved higher expression of glucose/fructose transporters.

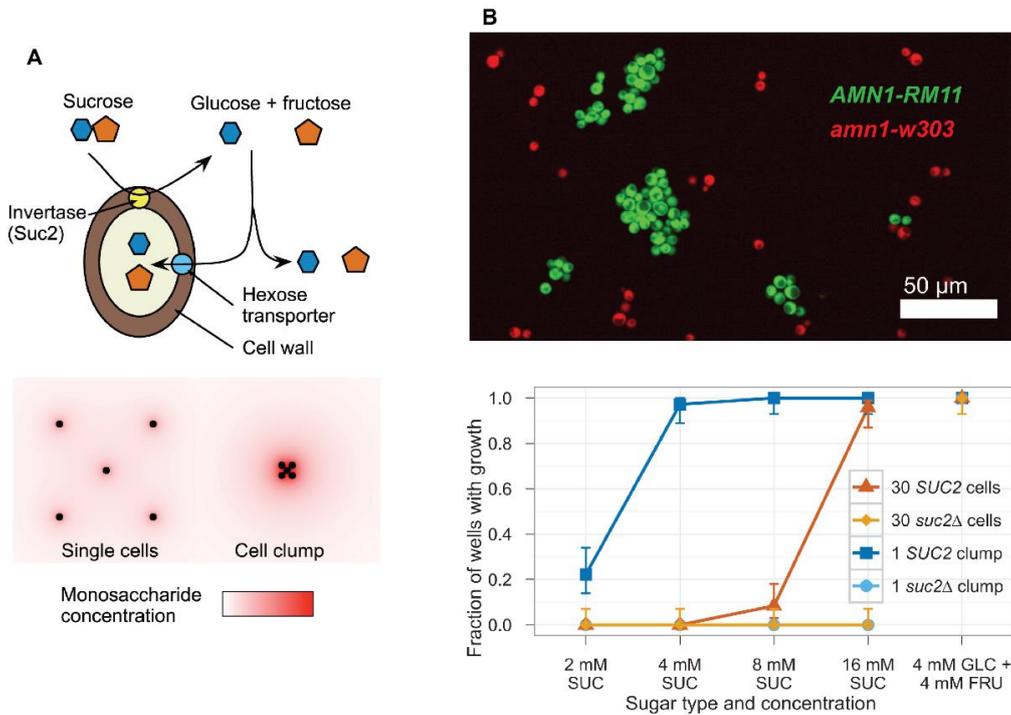


Figure 7 Yeast *Saccharomyces cerevisiae* as a model for the emergence of multicellularity. (A) Yeast need to convert sucrose to glucose and fructose via enzyme invertase. Local sugar concentration is higher if cells from aggregates than when they are alone. (B) Cells that form clumps can grow at lower concentrations of sucrose compared to single cells. Figure adapted from [37].

Travisano's group used gravity to select for, what they call, primitive multicellularity in otherwise unicellular yeast *S. cerevisiae* [27]. After rounds of selection in which they let the yeast liquid culture to settle, accompanied with gentle centrifugation, they selected for groups of cells that were able to make clusters via postdivision adhesion or by aggregation. They called it the snowflake yeast, as the clumps resembled snowflakes. The snowflake phenotype had a selective advantage, showing that it has a 34% higher fitness than individual cells under the same selection conditions. On the other hand, it suffered a 10% fitness cost in the absence of settling selection. They also claim that they observed division of labour within the snowflake yeast. While majority of cells remained viable and were able to reproduce, a minority of cells became apoptotic. These apoptotic cells act as break

points within multicellular clusters, which allows snowflake yeast to produce a greater number of propagules from a given number of cells. They think that this is functionally analogous to germ-soma differentiation, where cells specialize into reproductive and nonreproductive tasks.

Finally, in Palkova's group they looked at phenotype differentiation in yeast *S. cerevisiae* colonies [38]–[40]. They used dissection of the colonies grown for up to two weeks on agar containing glycerol and/or ethanol in order to observe the interior of the colony under confocal laser scanning microscope. They observed differential gene expression within colony, and a rather surprising differentiation between, what they call, U (upper) and L (lower) cells (Figure 8). Cell close to the gel and close to the top of the colony (U cells) look relatively healthy and as if they are in good nutrient conditions, while cells in the middle of the colony look exhausted and starving (L cells). They think that U cells on top of the colony feed on the nutrients made by L cells in the middle of the colony in the following way: L cells activate degradative processes that could, along with the activation of specific transporters, lead to the export of amino acids, sugars, and other metabolites. These compounds could then be used by U cells. Products of U cell metabolism (ammonia and possibly fusel alcohols, acetate, and pyruvate) are exported from U cells and reused by L cells and/or act as signalling molecules [38]. This nutrient channelling in yeast colonies reminds them of the nutrient channelling between liver, muscle, and tumour cells in mammals, so they suggest evolutionary conservation that might be exploited for uncovering new aspects of tumour biology.

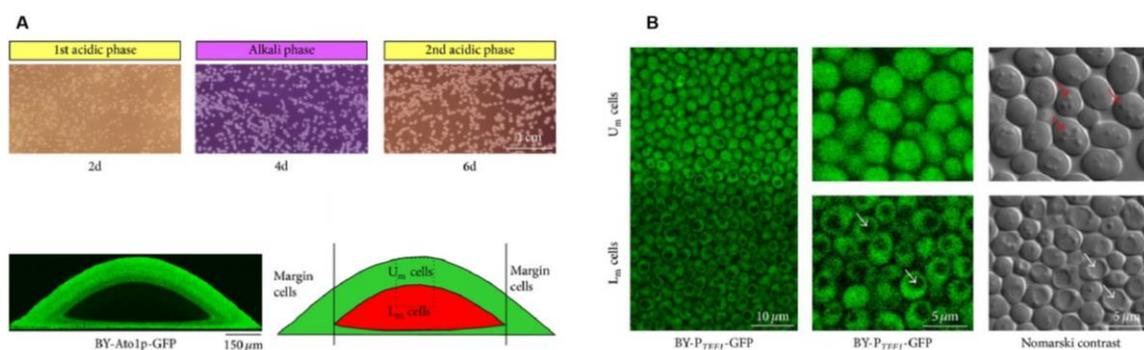


Figure 8 Differentiation of yeast *S. cerevisiae* colonies. (A) Colony growth goes through acidic and alkali phase during which colony differentiates to upper (U) cells and lower (L) cells. (B) Differentiated parts of the colony from up close. Difference in gene expression and cell morphology can be observed. Figure adapted from [40].

## **Yeast *Saccharomyces cerevisiae* – the eukaryotic model organism**

*Saccharomyces cerevisiae* is a superstar that goes by many names. It has accompanied human kind in some of the most important aspects of our existence. Indulgence in food (*Baker's yeast*), drinking (*Brewer's yeast*), and science (*Budding yeast*). As a nutritional source it served in production of foods that range from basic staples like bread or combating malnutrition for the most needy in our society [41], [42] to production of gold-plated Dom-Pérignon-infused rip-off extravaganza foods for the most greedy [43]. As a source of beverages we used it in the production of wine, beer, cider, and sake as far as 9000 years ago [44]. Work on the yeast *S. cerevisiae* has been awarded a Nobel prize five times since the year 2000. In 2001 for the discoveries of key regulators of the cell cycle, in 2006 for the studies of the molecular basis of eukaryotic transcription, in 2009 for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase, in 2013 for the discoveries of machinery regulating vesicle traffic, and in 2016 for the discoveries of mechanisms for autophagy [45]. The budding yeast is still an ample choice for scientists around the world and it will continue to be so, as argued in the review by Botstein and Fink about its use as a model system "*Yeast: An Experimental Organism for 21st Century Biology*" [4]:

*"However, in the intervening time, yeast, more than any other organism, has led the way to another, potentially more important frontier beyond the functions of single genes and proteins: the "systems level." The goal is understanding the functions of ensembles of genes and proteins as they act to maintain metabolism and cellular homeostasis under a great diversity of environmental conditions and to provide for the regulation and organization of reproduction, cellular growth, and development. For the foreseeable future, the experimental advantages offered by yeast will serve to keep this model organism at the forefront of this new frontier."*

*Saccharomyces cerevisiae* is a unicellular eukaryote. This means that it is composed of one cell only and that it has a real nucleus. In contrast to bacteria, which are just self-replicating bags of molecules, the budding yeast is a self-replicating bag of molecules with some compartmentalization inside. As one physicist once told me, yeast are bacteria, to the first approximation.

In the most common form, it has one set of chromosomes (haploid) or two sets of chromosomes (diploid), but sometimes even more than that (polyploidy), which gives it some additional properties in adaptation to different environments [46]. In my research, I use haploid yeast and I let them grow in a non-sexual life cycle. To divide from one cell into two, they need to go through the growth 1 (G1) phase, DNA synthesis (S) phase, growth 2 (G2) phase, and finally mitotic (M) phase in which they segregate their chromosomes, divide their nuclei, and at the end the daughter bud detaches from the mother cell (Figure 9). If from one yeast cell we get two yeast cells in about 90 minutes, which is a division time yeast can reach in nutrient rich conditions, this means that every yeast scientist every day is just about one week away from destroying the Earth. It would take just about a week for a single yeast cell ( $m \sim 100$  pg) to grow into a mass that is equivalent to the Earth's mass. For bacteria, like *Escherichia coli* ( $m \sim 1$  pg, division time  $\sim 20$  min), it would take less than two days [13]! However, this does not happen. The reason why this does not happen is the ecology and the environment. Just as scientists do not have an Earth-sized flask and an unlimited amount of nutrients (1. we do need to think about public spending, after all; 2. there is only one Richard Lenski [47]), the environment on Earth does not have unlimited resources, plus there are competitors which are in need for the same resources everywhere. On top of that, yeast and bacteria age and die too. Either from replicative aging (a limited number of divisions each cell can undergo) or chronological aging (length of time that a non-dividing cell survives) [48], [49].

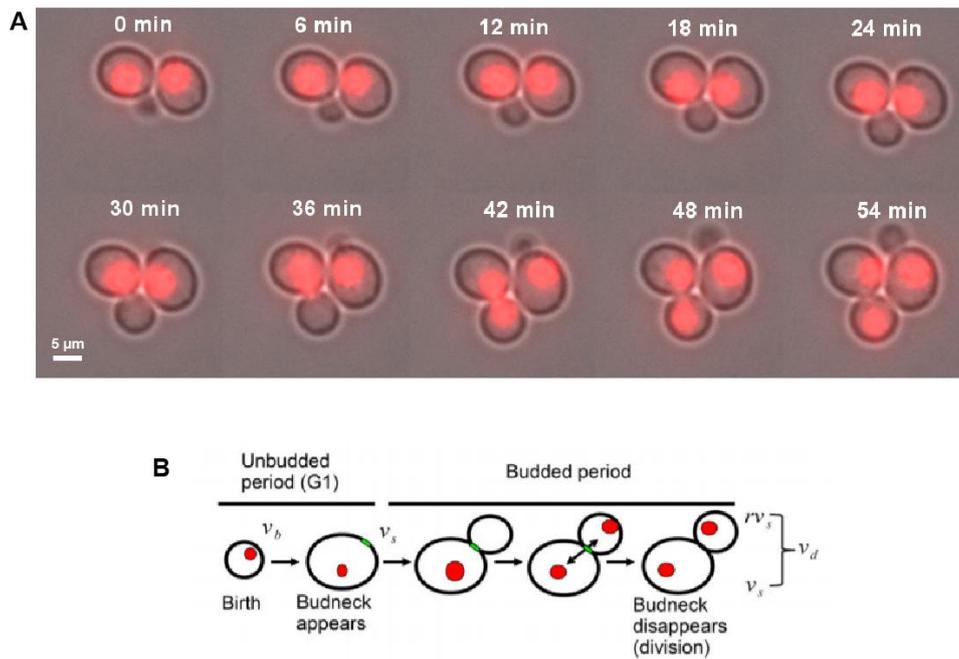


Figure 9 Growth of *Saccharomyces cerevisiae* cells. Nucleus is tagged with a red fluorescent protein. (A) At the beginning a bud is formed. Cells go through the G1, S, and G2 phase. At the end of the cell cycle, during mitosis (M phase), chromosomes segregate and nuclei divide. Adapted from [50]. (B) In a recent paper Soifer *et al.* proposed the “incremental model” of the budding yeast growth. Between two budding events a constant volume is added to a cell. When cell start to bud, only the bud grows. Adapted from [51].

This is the reason why we need to put *S. cerevisiae* in its natural ecological context first. Without it we might lose the insight into why and how yeast evolved the way they are.

## Ecology and socio-biology of *S. cerevisiae*

A common knowledge, often unreferenced, is that we can find yeast *S. cerevisiae* on grapes and other fruit. This is taken almost for granted as we make wine with the help of yeast. Other natural habitat that is often mentioned is the bark of oak trees. One habitat is rich with nutrients, especially with sugars, the other is nutrient poor. We domesticated many yeast strains through thousands of years of wine, beer, and bread making (Figure 10). There are also strains that infect humans, which are known as the clinical strains. And then there are the laboratory strains, a set of different strains that we took out of their natural environment and grew in our labs. Maybe the most common among them is the *S. cerevisiae* strain S288c. In

the review article “*Natural history of budding yeast*” by Duncan Greig and Jun-Yi Leu, every sentence on the relationship between the natural habitat and the transfer to the lab is very much on point and well written, so I have no other choice than to quote the paragraph in its entirety [52]:

*“In 1996, the most popular yeast clone, S288c, became the first eukaryote to have its entire genome sequenced. Thus began the current era of yeast genomic high technologies. Can we now look back and congratulate ourselves that we know the biology of yeast better than that of anything else? Is it just a matter of time and technology before we know everything we need to know about yeast? Not really. The S288c clone was made by crossing a strain found on a rotting fig in 1938 with a number of others, including brewing and baking strains, to produce an offspring with properties useful for the lab. This strain has since been domesticated in a laboratory environment that is, one imagines, about as different from its natural environment as it is possible to get. It is imprisoned, starving and chilled (or even frozen solid) for long periods, and then thrown into a warm sweet soup that is enriched with carcasses of its own species (‘yeast extract’). Without competition from other organisms (which microbiologists call contaminants), it grows rapidly to colossal density before a single individual, perhaps a useful mutant or transformant, is plucked out from the crowd and saved. This has had strange consequences for evolution. Deleterious mutations that would normally be removed by natural selection can accumulate in lines going through such population bottlenecks, even if they reduce fitness. Traits that are probably important in the wild but undesirable in the laboratory, such as clumpiness that helps cells stick together to survive environmental stress or preference for some potential mates over others, may be selectively removed. Other traits may deteriorate because they are rarely needed in the lab environment. The extent of this problem is illustrated by the fact that scientists working on sex avoid S288c, which can barely be persuaded to do it, in favour of wilder strains... Our lab-centric view, coming from decades of studying dividing cells, that stationary phase cells are just passively awaiting food now seems overly simplistic. In nature it seems more likely that yeast spend most of their time in low nutrient conditions, and adaptations to this environment might be more important for yeast evolution than the high growth rates that laboratory experiments typically focus on.”*

There are currently two prominent theories about the natural ecology of the budding yeast, apart from the too simplified view that we can find them on only on grapes and oak trees. The first one developed by Goddard and Greig states that the

yeast *S. cerevisiae* is a generalist species, or as they put it – nomadic [53]. This means that it is not adapted to any particular niche or environment, but rather that we can find it across many habitats in low frequencies, with some notable exceptions when environmental conditions are very beneficial. Their first argument is that the method for sampling budding yeast from the wild is flawed as it might favour the proliferation of *S. cerevisiae* at the cost of outcompeting other microorganisms in a sample. This can also work the other way around. The sampling methods might favour other microorganisms that might outcompete the budding yeast. In both cases we would either overestimate or underestimate *S. cerevisiae* abundance in the wild. They also claim that there is evidence of the budding yeast being very rare on fruit, even in vineyards. A recent metagenomic sequencing study across different vineyards in New Zealand found *Saccharomyces* species at a frequency of only 1:20000 of the fungi found on ripe grapes [54]. Previous studies have shown that other yeast dominate the early stages of wine fermentation, and *S. cerevisiae* becomes abundant only after several days [55]. When wine is spontaneously fermented, what often happens is that very little ethanol is produced because no fermentative microbe dominates the mix [56]. Another argument for *S. cerevisiae* to be a nomad is that it is not found only associated to wine, bread, and beer but it is also isolated from other fruits, insects, humans (as a commensal or pathogen), soil, various plants, and from oak trees. In addition to that, they argue that *Saccharomyces cerevisiae* has a rich metabolism that enables it to survive or grow in a wide range of environments with varying nutrient availabilities. These environments include both low and high carbon and nitrogen concentrations, strongly acidic ~pH 3 to alkaline ~pH8 conditions, survival in water but also at high osmolarity in NaCl concentrations of at least 1.3 M, temperatures ranging from close to 0 to around 45°C. They think that this evidence suggests that the set of conditions in which *S. cerevisiae* is found to survive is very broad and in line with its proposed generalist nature. As a last argument they present the fact that only 20% of ~6000 genes are necessary for growth in simple laboratory medium [53]. Goddard and Greig propose that their Nomad Model should be used as a null hypothesis when evaluating adaptive explanations and not immediately assuming that *S. cerevisiae* is adapted to a specific niche.

Almost at the same time, Goddard and Knight proposed an additional theory, called “fruit forest-reservoir hypothesis”. In this theory, *S. cerevisiae* thrives on fruit during the fruiting season, but when the season is over, it is transferred to soil or tree bark via vector insects. Since nutrients are scarce in such environment, cells sporulate and wait in this dormant state for the next fruiting season, when they are

again transferred back to fruits via vector insects. Yeast have been isolated from bees [57] and social wasps [58], which may serve as vectors. In addition to that, it seems like they have a special relationship with another model organism, the fruit fly *Drosophila melanogaster*. It has been shown that *S. cerevisiae* spores can survive in the digestive tract of the fruit fly [59]. Moreover, there is a conclusive body of evidence that the budding yeast produces several secondary metabolites which attract the fruit fly [60]–[63]. Therefore, the fruit fly could serve as a vessel between different habitats and yeast might have evolved in a way to better attract the fruit fly as higher attraction would lead to a higher dispersal and this might increase the chance of proliferation and survival of the budding yeast. To reconcile the Nomad hypothesis and the fruit forest-reservoir hypothesis, authors suggest that indeed Nomad hypothesis might be true and that yeast have the ability to survive in many habitats, existing as spores in most of them.

Doing a quick search of the word “Saccharomyces” in PubMed results in more than 120000 hits, “Ecology” results in 140000 hits, while a search of “Saccharomyces AND Ecology” gives just around 500 hits. It is clear that we lack a proper understanding of the ecological context of *S. cerevisiae* and that only a small number of groups are tackling this issue. If Dobzhansky is right that “*Nothing in biology makes sense except in the light of evolution*” [64], and ecology and environmental context are tightly connected to evolution, than we need to put more effort in understanding the budding yeast’s ecological context because it might help us understand why *S. cerevisiae* evolved the complex molecular machinery the way it did, which I will review next. It will become clear that, irrespective of the huge progress, we still lack some basic insights, which might be better understood if we would better understand the budding yeast’s natural environment.



Figure 10 651 budding yeast strains isolated from different sources. The strains that are missing to complete the picture are the clinical isolates which infect humans. Figure adapted from [44].

## Metabolism of *S. cerevisiae*

Metabolism encompasses all chemical transformations that take place in cells. Its role is to maintain life through conversion of nutrients into energy needed for cellular processes, production of key building blocks of life such as amino acids, lipids, carbohydrates, and proteins, and elimination of harmful or useless wastes (Figure 11). In yeast *Saccharomyces cerevisiae* it is a complex network of 1175 metabolic reactions and 584 metabolites that interact, adapt to the environment and to the needs of cells, and ever change to sustain growth, survival and homeostasis [65]. Its role is so fundamental to life that many of its core aspects have been conserved through evolution, and we can find them from bacteria and yeast to human beings [66]. This implies a conservation that lasted almost three billion years, as our last universal common ancestor (LUCA) is supposed to have been living around three billion years ago [67]. As a matter of fact, some aspects of metabolism are so conserved that we can replace them in yeast with their human counterparts with up to 92% replaceability, meaning that human genes code for proteins that can sustain life in yeast, as it was demonstrated in 2015 by Kachroo and colleagues when they replaced more than 400 genes in *S. cerevisiae* with their human orthologs [68]. In 2017 they also demonstrated high replaceability of metabolic genes (for some parts of the metabolism up to 100%) between *S. cerevisiae* and bacteria *Escherichia coli* [69].

In order to grow and divide, cells need to take up the nutrients from their environment, usually through membrane transporters, and transform them into usable products. Enzymes are the key players in this metabolic process. They catalyse the reactions of transformation from one metabolite to another. Some of the metabolites are used for energy, some for building other molecules, some are used for signalling, and some are excreted back to the environment. We can imagine cells as very efficient processing units which act upon an input from the environment and process it into products and output. This efficiency is visible in metabolic processes where cells tend to keep optimally low concentrations of metabolic pools, but due to the quick turnover time at a scale of seconds they can renew them completely in a little amount of time [70]. This is especially important for quick adaptation as cells are continuously exposed to perturbations in their natural environment and need to adjust the rates of synthesis and consumption of metabolites. To coordinate this myriad of reactions, cells have evolved control mechanisms, signalling pathways that have the ability to dynamically process information and quickly react to change [71]–[73].

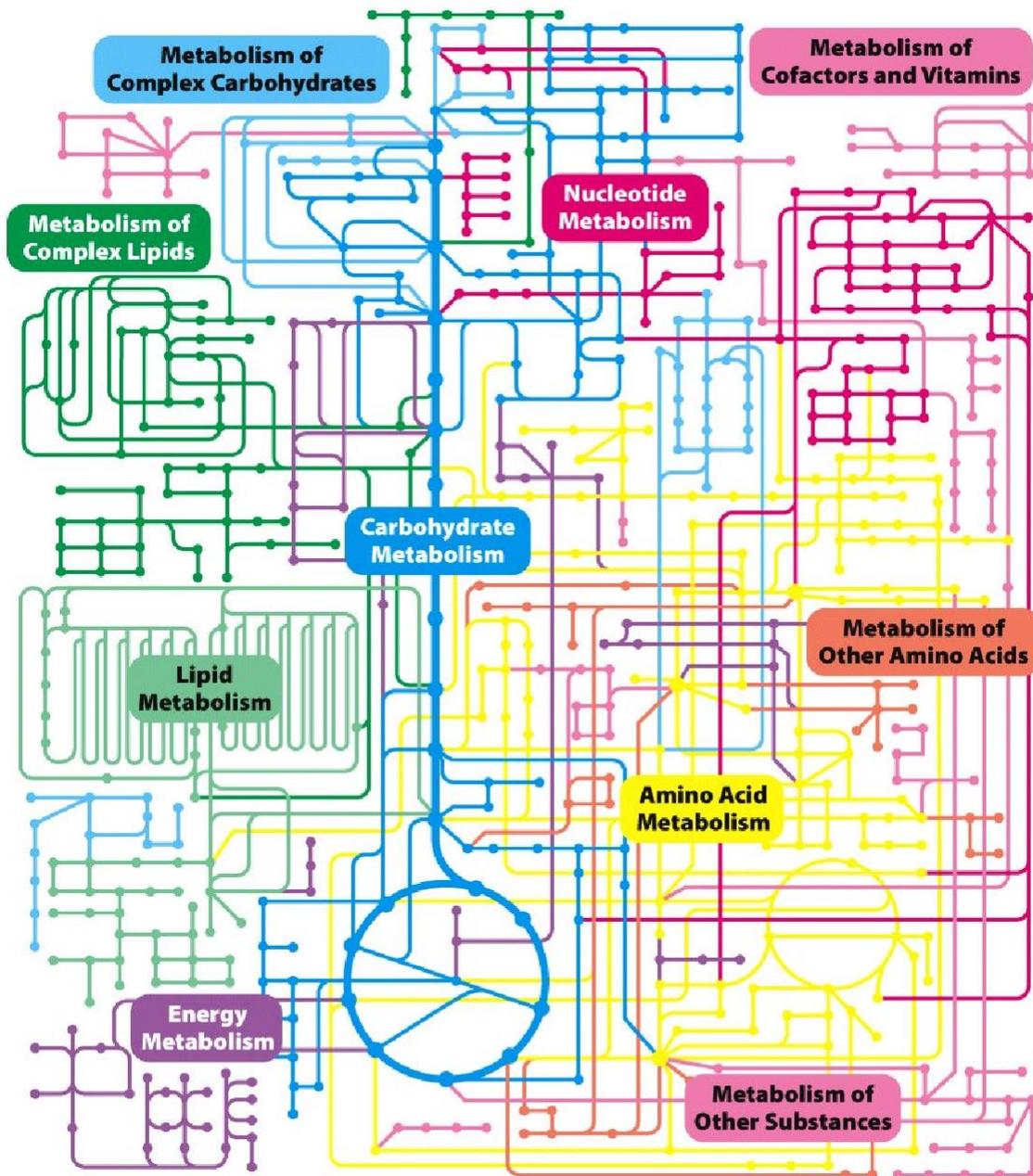


Figure 11 A simplified map of metabolism. Each colour represents a specific major pathway with bold circles representing an intermediary product. Figure adapted from [74].

Metabolism and metabolic phenotypes are just one part of the picture that governs cell function. A layer above it are genes which hold the vital information about cellular identity. They store the information on which type of proteins can be expressed in cells. As such, they are highly regulated through a collection of molecular regulators that form a gene regulatory network (Figure 12). Visualisations of gene regulatory networks and interactions are as complex as metabolic networks since both of them consist of many actors with many

interactions. Costanzo *et al.* were able to generate a quantitative genetic interaction profiles for ~75% of all genes in *S. cerevisiae*. Their genetic interaction map was constructed by examining 5.4 million gene-gene pairs which revealed a functional map of the cell in which genes that code for similar biological processes cluster together and delineate specific pathways which define gene function. From this network they were able to identify functional cross-connections between all bioprocesses and map a cellular wiring diagram of pleiotropy (one gene influencing two or more seemingly unrelated phenotypic traits) [75]. The regulators in this network can be DNA, RNA, and proteins. If a protein (encoded by the gene) controls transcription rates of other genes it is called a transcription factor. They can control, activate and repress, other genes, alone or often in interaction with other proteins. Proteins themselves are also regulated through molecular signals or marks. They can be activated or inactivated through processes like (de)phosphorylation, which is the most common post-translational modification. For example, almost half of the enzymes in *S. cerevisiae* are phosphoproteins [76]. The complexity and abundance of interactions can even sometimes lead to challenges in nomenclature as demonstrated by enzymes that catalyse phosphorylation, called kinases. For example, MAPK pathway consists of MAP kinase (MAPK), kinase of the MAP kinase (MAPKK), and kinase of the kinase of the MAP kinase (MAPKKK). And even MAPKKKK exists. In a biochemistry class this would usually be time for a brake.

Kinetics and time scales of these processes also need to be taken into account as, for example, gene expression can take minutes or hours to have an effect, while enzymes and transporters can process hundreds and thousands of molecules per second. Cells have evolved all these regulation mechanisms to cope with inevitable dynamics of living systems in everchanging environments. They need to actively sense, signal, and process internal and external stimuli through a network of interactions and feedbacks to produce an output, a reaction, a phenotype, a changed behaviour and structure that gives rise to a cellular function adapted to a specific moment in space and time.

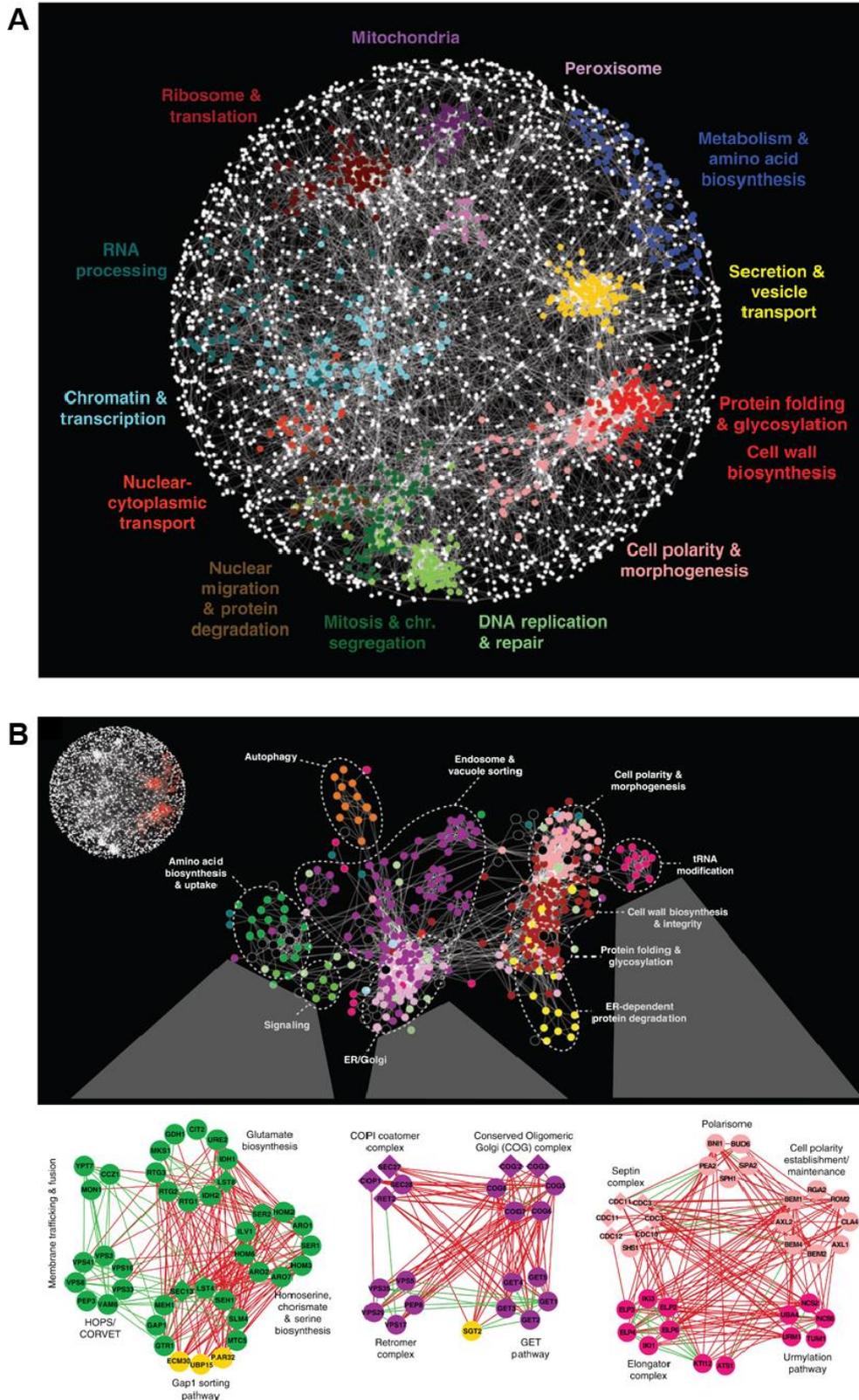


Figure 12 Gene regulatory network of *S. cerevisiae*. Genes are represented as dots and edges represent interactions between genes. Genes that have similar patterns of genetic interactions cluster close to each other. (A) A zoom-out view of interactions and their function. (B) A zoom-in to specific parts of the interaction map where individual processes or genes can be seen to interact. Figure adapted from [75].

### ***Glucose sensing, signalling and transport***

All gene regulation and metabolic networks rely on energy that comes from carbon sources. In my research I mainly focused on the glucose metabolism of yeast. Glucose is by far the most preferred carbon source of yeast *S. cerevisiae*. In fact, it is so preferred that a whole signalling network exists which role is to represses the expression of genes that are involved in the processing of other carbon sources, like galactose, maltose, sucrose, and ethanol. Glucose is a starting point that involves many cellular processes and its use is well conserved across species (Figure 13). First, glucose is processed through an oxidative process called glycolysis that ends with a production of 2 molecules of pyruvate and 2 molecules of ATP, which is used as the main source of energy and is often regarded as the “energy currency” of a cell as it can be “spent” to catalyse many vital reactions. Cells can also store glucose as glucose disaccharide trehalose and glucose polysaccharide glycogen, which can be converted back to a glucose monosaccharide. Glycerol is also one of the products of glucose catabolism which can be converted back to glucose, but it is also a key player in cell response to an osmotic shock as it is used to balance the osmotic pressure. Cell wall synthesis and other pathways, like pentose phosphate pathway are tightly linked to glucose.

When pyruvate is produced, cells can enter into two different modes of metabolism. They can either ferment or respire. Through fermentation, cells produce carbon dioxide, ethanol, and 2 molecules of ATP. They also produce acetate which can “re-enter” into metabolism and be processed further as a source of energy. On the other hand, through respiration, which starts when acetyl-CoA (produced from pyruvate or acetate) enters into the citric acid cycle, cells can produce a maximum of 36 ATP molecules, carbon dioxide, and water. From many of these steps additional vital products are produced, like fatty and amino acids. Some of these steps are reversible, meaning that cells can produce intermediary metabolites back from the final products, all the way up to glucose through the process of gluconeogenesis. Many of these products, like glucose, amino acids, glycerol, acetate, and ethanol can be taken up from the environment through membrane transporters and channels.

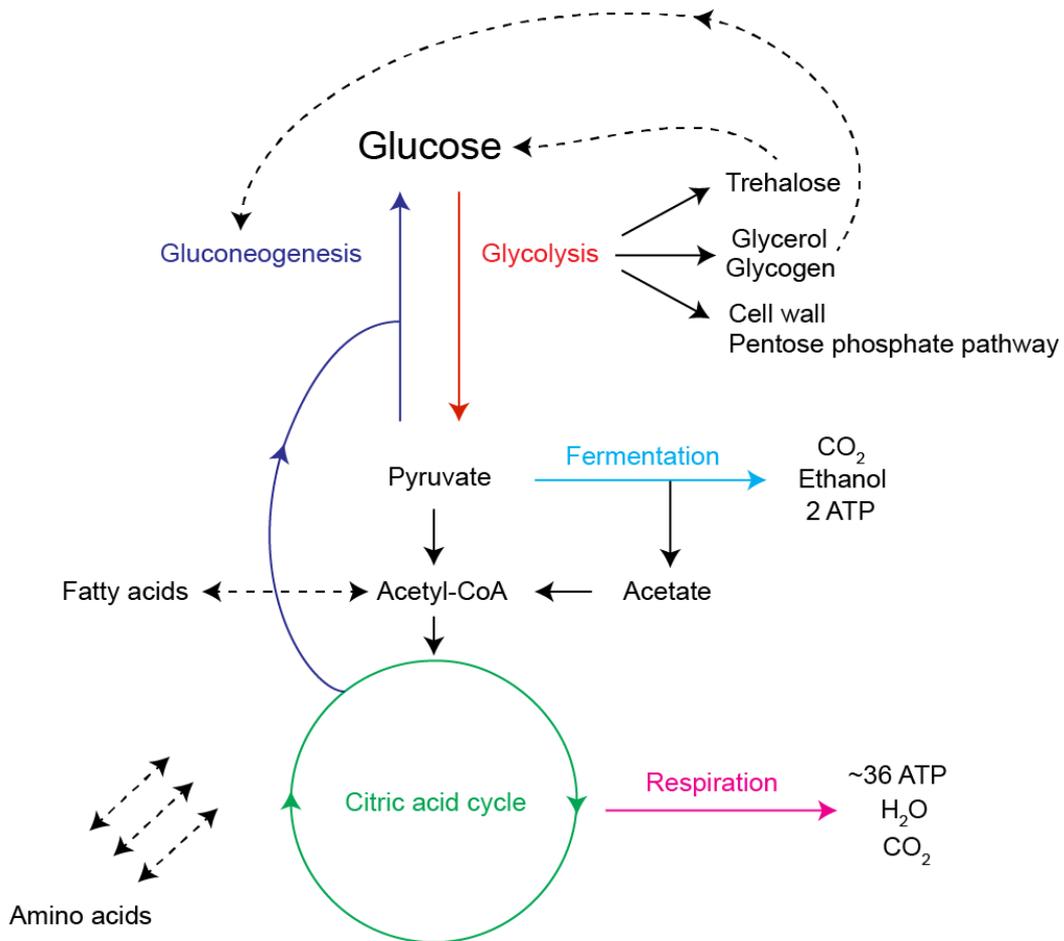


Figure 13 A simplified scheme of glucose utilization in (yeast) cells. Glucose, as the preferred carbon source, is the starting point of many metabolic reactions in cells. It is catabolized through glycolysis but it can be also anabolised through gluconeogenesis. Intermediary products of glucose are used in downstream reactions of fermentation and respiration, as well as building blocks for other vital molecules.

### ***To ferment or to respire? The Crabtree effect***

Usually, respiration is referred to as aerobic and fermentation as anaerobic since oxygen is in most cases needed as electron acceptor in the electron transport chain during the process called oxidative phosphorylation. Fermentation, on the other hand, usually happens in anaerobic conditions. However, there are exceptions to this case. One of those exceptions is the yeast *S. cerevisiae*. It prefers to ferment when glucose concentrations are high regardless of the presence of oxygen. This phenomenon is called the Crabtree effect, after the English biochemist Herbert Grace Crabtree [77]. Interestingly, it is a very similar phenomenon to the Warburg effect in cancer cells, where cancer cells tend to ferment glucose instead of respire in the presence of oxygen [78]. What is strange about this phenomenon is that cells

produce much more molecules of ATP through respiration, than through fermentation. In yeast *S. cerevisiae* it is estimated to be up to 9 times more [79]. There are two potential explanations for the reason why the Crabtree effect would evolve and which benefits it could have. The first one is the so-called rate/yield trade-off hypothesis which makes a difference between ATP production rate and ATP yield [80]. ATP production rate is the amount of ATP produced per unit of time, while ATP yield is the amount of ATP produced per unit of substrate. Therefore, ATP can either be produced fast, at a high rate but low yield, or it can be produced efficiently, at a low rate but a high yield. This would mean that fermentation is much faster and provides enough resources to proliferate at high glucose concentrations compared to slow respiration. The second explanation is based on the theory of niche construction, which states that organisms can construct their environment through their activity. In the words of Jack Nicholson in Scorsese's *"The Departed"*: *"I don't want to be a product of my environment. I want my environment to be a product of me"*. The idea here is that yeast *S. cerevisiae* interfere with their competitors in the environment by producing ethanol via fermentation which they can withstand in higher concentrations but other microorganisms like bacteria cannot. On top of this cells could afterwards aerobically respire ethanol as a source of energy, but it is unclear how much of it is conserved in natural habitats. They could also change the pH of the environment through acidification and even heat produced during fermentation might have some influence on the temperature-sensitive organisms [55]. Some researchers have suggested that the emergence of the Crabtree effect coincides with the emergence of high sugar environments as flowering plants became widespread on Earth around the same time. Therefore Crabtree positive yeast might have evolved this adaptation to feast on nectar and fruits of these plants [81]–[83]. This hypothesis probably has the most flaws since the Crabtree effect probably evolved before the emergence of flowering plants in several yeast lineages and its evolution took a long time which just happened to coincide with the emergence of flowering plants [84]. Moreover, although it is true that fruit environments can have high sugar content, e.g. around 20g/100mL in grape [85], fermentation in presence of oxygen can be induced at concentrations of glucose as low as 15mg/100mL (0.015% glucose) [86].

The reason why has the Crabtree effect evolved are still under debate and no conclusive answers can be given. However, recent research from Kowallik and her colleagues has shown some surprising and promising results [87]. Since most of the research on Crabtree effect is done in sterile laboratory conditions on standard media, they wanted to bring the experiments closer to what is happening in natural

environment. They used *S. cerevisiae* strain that could normally ferment and made a *S. cerevisiae* mutant that did not exhibit Crabtree effect, it was Crabtree negative. They grew them either alone or together in standard laboratory media and in grape juice, as well as in competition with other microorganisms that can be found in natural environment. In laboratory medium, the Crabtree positive strain had lower yield compared to the Crabtree negative strain when grown separately in a monoculture. It did, however, have a higher fitness when directly competed with the Crabtree negative strain in a coculture. Surprisingly, the fermenter was less fit compared to the respirer both alone and in a coculture in a grape juice. This changed when microorganisms commonly found on grapes were added to the mix. In this case the fermenter outcompeted the respirer, in some cases its yield was 1600 times higher. Sometimes the respirer even died. The fitness relative to each other also depended on the composition of the grape microorganisms. They concluded that Crabtree positive strain probably has advantage due to better interference competition and that the Crabtree effect indeed might be an adaptation to sugar rich environments. This also shows that the ecological context is very important, and that we have to understand it to be able to interpret genetic and physiological differences between strains.

### ***Glucose transport***

Cells do not change their environment only by secreting molecules, like ethanol, they also do it by taking up nutrients from the environment. When it comes to glucose import, *S. cerevisiae* has evolved a set of 20 hexose transporters, HXT1-17, GAL2, SNF3, and RGT2 [88]–[90]. They can transport glucose, fructose, mannose, and galactose (GAL2) via facilitated diffusion, which is a passive transport process through transporters on the membrane, meaning that it needs no direct energy investment from molecules like ATP. They are all closely related and relatively conserved proteins which have 12 membrane spanning domains that do, however, differ at their amino- and carboxyl- terminal tails which are both located in the cytosol. None of them individually is essential for survival and growth [91], and when individually overexpressed in a strain that lacks all glucose transporters they do support growth on glucose with variable efficiency [92], [93]. Exceptions are HXT12 which is probably a pseudogene that lost its function, and SNF3 and RGT2 which evolved into sensors of extracellular glucose and can no longer transport it [94]. Transporters HXT1-HXT7 are considered to be metabolically most relevant as they are expressed at significant levels in standard laboratory conditions, while

other glucose transporters tend to be expressed in more specific environmental conditions and still remain under investigation [95], [96]. It is still unclear as to why would *S. cerevisiae* need so many transporters, but in general it is thought to be an adaptation to all varieties of environments that they can encounter [81]. Each of the seven major glucose transporters is expressed in specific glucose concentrations and each one of them has a specific uptake kinetics and specific substrate affinities [91], [97], [98]. We can roughly split them in three categories: low-affinity, medium-affinity, and high-affinity transporters. The kinetics of transport follows Michaelis-Menten like kinetics, meaning that transport reaches a saturation point as substrate concentration increases. This is reflected in the Michaelis constant ( $K_m$ ) which represents the concentration of substrate at which the reaction reaches its half maximum rate. If  $K_m$  is high, transporter has a low affinity for glucose as high concentration of glucose is needed to reach the half maximum reaction rate. Conversely, if  $K_m$  is low, transporter has a high affinity for glucose and reaches saturation point in lower concentrations of substrate.

HXT1 and HXT3 are low-affinity transporters which have  $K_m$  of  $\sim 100$  mM and 30-60 mM respectively, and their maximum uptake rate ( $V_{max}$ ) is between 30 and 50 nmol glucose  $\text{min}^{-1}$   $\text{mg}^{-1}$  wet weight of yeast. They are expressed in high glucose concentrations. HXT2, HXT4, and HXT5 are medium-affinity transporters which have  $K_m$  of 5-10 mM and  $V_{max} \sim 15$  nmol. $\text{min}^{-1}$ . $\text{mg}^{-1}$ . HXT2 and HXT4 are expressed in medium to low glucose concentrations, and HXT5 is expressed during growth arrest. HXT6 and HXT7 are high-affinity glucose transporters which have  $K_m$  of  $\sim 1.5$  mM and  $V_{max} \sim 10$  nmol. $\text{min}^{-1}$ . $\text{mg}^{-1}$ . They are expressed in very low glucose concentrations and differ only in two amino acid residues, but they do have different promoters.

HXT2 is somewhat peculiar as its transport kinetics seems to be influenced by the growth conditions. Cells that are grown in high 100 mM ( $\sim 2\%$  glucose) glucose have a  $K_m$  of  $\sim 10$  mM, but if they grow in low glucose conditions the transport kinetics shows both high-affinity  $K_m$  of  $\sim 1.5$  mM and low-affinity  $K_m$  of  $\sim 60$  mM. It looks like HXT2 substrate affinity is modulated in a glucose dependant manner [96].

It is critical to stress out here, as it will become even more obvious throughout this manuscript, that glucose uptake is a highly dynamical process which tightly depends on transient glucose concentration in the immediate environment and that it is hard to understand and capture the dynamics of glucose uptake of the whole cell, since it is a highly regulated and, still in some critical aspects, unknown process. All of the kinetics experiments are done in static glucose concentrations,

most often a single high and a single low concentration, and they might not reflect the full complexity of glucose transport.

### ***Glucose sensing and signalling***

There are three main pathways that we know of which are regulated by the presence of glucose in the extracellular and intracellular environment. We can distinct them from each other but they do interact through a very complex interplay of intracellular gene and metabolic networks. They constantly adapt to the glucose concentration, and thus are very dynamic.

The first one is the Snf3p/Rgt2p-Rgt1p pathway which is based on Snf3p and Rgt2p glucose sensors which have a very similar structure to glucose transporters and they probably lost their transport function through evolution (Figure 14). Snf3p is thought to be a high-affinity glucose sensor which responds to low glucose concentrations and consequently regulates the expression of medium to high affinity glucose transporters, while Rgt2p is thought to be a low-affinity glucose sensor which responds to high glucose concentrations and therefore regulates the expression of low-affinity glucose sensors. Snf3p is also sensitive to fructose, mannose, and some glucose analogues. Both sensors have particularly long C-terminal tails located in the cytoplasm and it is believed that they are the main interaction points for downstream regulation. When specific residues of these sensors are mutated or the long C-terminals are deleted, their ability to induce the expression of glucose transporters is lost, but it has also been found that some mutations cause constitutive expression of glucose transporters even in absence of glucose. If the tails are transplanted to a glucose transporter, glucose induction signal is partly restored. Also, expression of isolated C-terminal tails leads to a constitutive glucose signal. However, the need for tail is not absolute as it has been shown that overexpression of a tailless Rgt2p can still preserve its signalling function. Therefore, it is thought that determinants of glucose sensing are found in the 12 membrane-spanning domains of the sensors, while the long tails may serve as some kind of amplifiers of the induction signal.

So far no one has demonstrated that a specific ligand binds to the glucose sensor but the current propositions state that the binding of glucose leads to conformational changes in the sensor. There are several important downstream proteins which transduce the glucose signal. First, there are two co-repressor Mth1p and Std1p which interact directly with the sensors. It seems like Mth1p maintains

the repression of glucose transporter genes in the absence of glucose, while Std1p establishes transporter repression during transition to glucose exhaustion. In absence of glucose constitutively expressed transcription factor Rgt1p is bound to the promoter of HXT genes and no expression can go forward. Mth1p and Std1p bind to Rgt1p to maintain the repression of HXT genes. However, when glucose is present, Mth1p and Std1p are phosphorylated by casein kinases I, Yck1p/Yck2p, which serves as a signal for SCF<sup>Grr1</sup> complex to mark them for ubiquitination and subsequent degradation by proteases. Since Mth1p and Std1p are degraded, they do not bind to Rgt1p anymore and Rgt1p becomes exposed to phosphorylation mediated by the cAMP/PKA pathway which is also one of the pathways sensitive to glucose. Rgt1p can also serve as a transcription activator of HXT1 expression in high glucose, probably through its distinctive property of differential phosphorylation pattern mediated by the cAMP/PKA pathway. There has recently been a challenge to the view that Yck1p/Yck2p and Mth1p/Std1p directly interact, therefore it has been suggested that there might be one more unknown signalling component in this process. The Snf3p/Rgt2p-Rgt1p pathway seems to be sensitive and responsive to subtle changes in corepressor levels as just enough corepressor concentration is maintained to repress their targets, which also facilitates rapid induction of expression in the presence of glucose. In addition to all of this, Rgt1p activity seems to also be influenced by Snf1p, which is the main actor of yet another pathway involved in glucose signalling. Specific experiments which led to all these conclusions can be found in the following four recent reviews by the teams who have been working on glucose sensing and signalling for more than two decades [73], [95], [96], [99].

## Glucose induction pathway via Snf3p/Rgt2p-Rgt1p

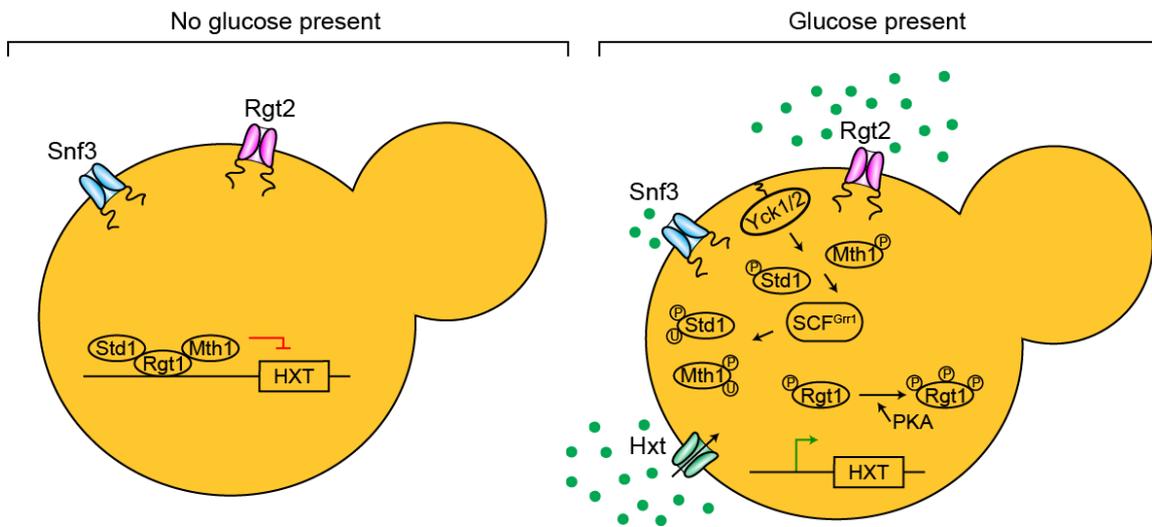


Figure 14 Snf3p/Rgt2p-Rgt1p glucose induction pathway. When there is no glucose in the environment, corepressors Std1 and Mth1 are bound to the transcription repressor Rgt1 which represses HXT glucose transporter expression. When glucose is present, Snf3 and Rgt2 sensors mediate a response which final result is degradation of Std1 and Mth1 corepressors and a release of Rgt1 which is phosphorylated by the cAMP/PKA pathway. Hence, glucose transporters are expressed. Figure adapted from [96].

The second major pathway sensitive to glucose availability is the Snf1p/Mig1p pathway. It is the most important glucose repression pathway which affects the expression on many genes, including the ones involved in the utilization of carbon sources other than glucose, gluconeogenesis, respiration, and some of the glucose transporters. In a nutshell, if cells do not want to waste their cellular resources on production of unnecessary genes when glucose, the preferred carbon source is present, they use this pathway to shut them down. This pathway is also involved in the release of glucose repression upon glucose depletion. The way glucose actually triggers this pathway and through which signal transduction is not very clear. What is clear, however, is that in order to generate the signal, glucose needs to be transported and phosphorylated, but not further metabolised. Phosphorylation of glucose is catalysed by hexokinase 2 (Hxk2p) in the first step of glycolysis. Interestingly, in addition to this metabolic function, Hxk2p has a glucose repression function as it localizes to the nucleus to repress other genes in coordination with Mig1p transcription factor. It seems like that there is no correlation between the phosphorylation and glucose repression function of Hxk2. Glucose repression correlates well with glucose transport activity and glycolytic flux. When glucose uptake is impaired but the extracellular glucose concentrations are high, cells trigger glucose repression according to the glucose uptake and not

according to the extracellular glucose concentration. For example, if glucose uptake in a genetically manipulated mutant corresponds to the wild type uptake at 0.2% (11 mM) glucose, glucose repression will proceed as if the cell is in 0.2% glucose environment, and not in 2% glucose environment in which the mutant is put to grow.

Glucose repression through this pathway proceeds in the following, simplified, way (Figure 15). When glucose is present in the environment, Snf1p kinase is inactivated and it cannot phosphorylate Mig1p. Dephosphorylated Mig1p thus stays in the nucleus and binds to the promoter of glucose repressed genes, rendering them inactive. However, when glucose is depleted, Snf1p is activated and it phosphorylates Mig1p which is then excluded from the nucleus and localized to the cytosol. This releases the repression of the glucose repressed genes and their expression can be induced. In addition to Mig1p, yeast *S. cerevisiae* has repressor Mig2p which is not inactivated by Snf1p nor its expression is repressed by glucose. It is always located in the nucleus and acts synergistically with Mig1p. There is no evidence so far that it represses genes alone, unlike Mig1p. Among other genes, Mig2p represses some of the glucose transporters and the relative contribution of Mig1p and Mig2p in repressing them has been found to depend on glucose concentrations. Specific experiments which led to all these conclusions can be found in the following four recent reviews [73], [95], [96], [99].

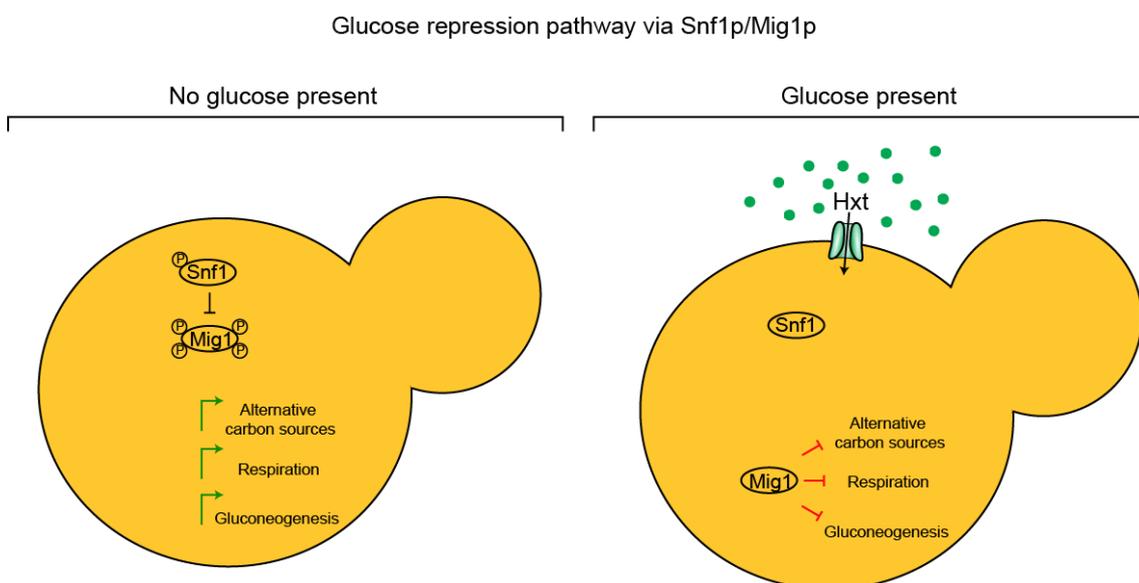


Figure 15 Simplified scheme of Snf1p/Mig1p glucose repression pathway. When no glucose is present, activated Snf1p kinase phosphorylates Mig1p which is then localized to cytosol. This releases repression from glucose repressed genes. When glucose is present, inactive Snf1p cannot phosphorylate Mig1p which stays localized to the nucleus and represses the expression of glucose repressed genes.

Glucose induction pathway via Snf3p/Rgt2p-Rgt1p and glucose repression pathway via Snf1p/Mig1p are not independent from each other. As a matter of fact, they are tightly interconnected through many common mediators (Figure 16). It is a network of at least eight regulatory genes that form auto-regulatory and cross-pathway-regulatory loops which govern the expression of HXT and other genes. This leads to four different scenarios, regulation of glucose induction by glucose repression, regulation of glucose repression by glucose induction, autoregulation of glucose induction, and autoregulation of glucose repression. It is speculated that such complex interaction between these pathways arose as a way to provide a graded derepression of different glucose transporters in response to different glucose concentrations so that only most appropriate transporters are expressed as a function of the transient glucose availability in the environment [96].

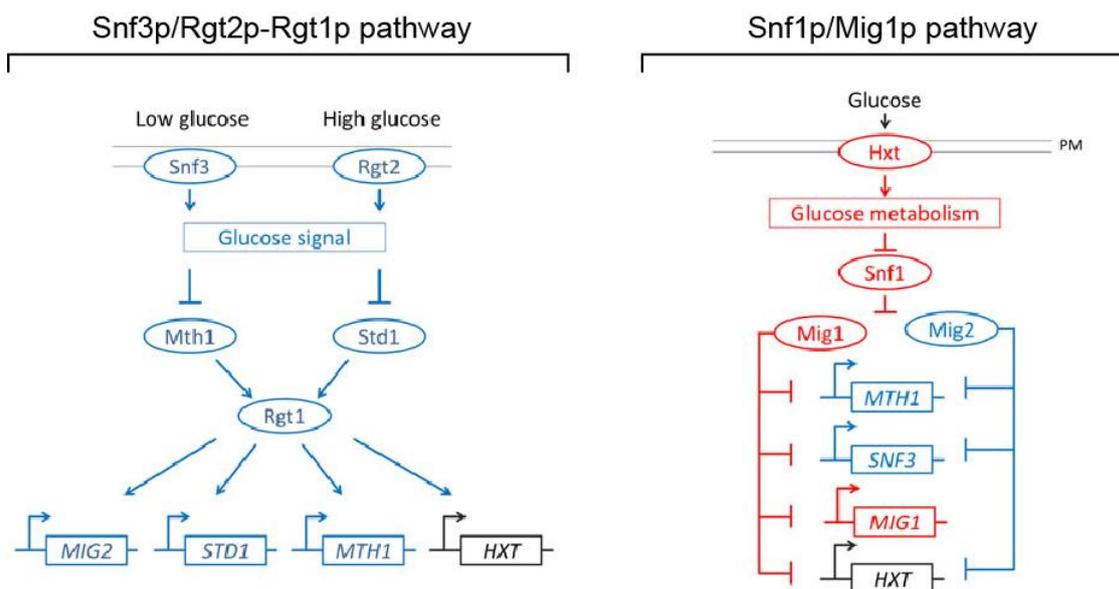


Figure 16 Scheme of interactions between glucose induction and glucose repression pathways. Many genes are shared between the two pathways and lead to cross-regulation and auto-regulation. Figure adapted from [96].

The third major pathway sensitive to glucose is the cAMP/PKA pathway. This pathway is involved in many physiological processes in cells like growth, proliferation, metabolism, stress response, aging, morphogenesis, and development, all according to nutrients availability [95]. Activation of the protein kinase A (PKA) is connected to dramatic changes in transcriptional identity and biosynthetic

activity of the cell. It is activated when glucose is present and cells enter into fermentative metabolism. In contrast, if any of the essential nutrients is absent, cells enter into growth arrest and stationary phase which leads to the downregulation of the cAMP/PKA pathway (Figure 17 B). There are two components that regulate this pathway (Figure 17 A). The first one is the G-protein coupled receptor (GPCR) system which senses extracellular glucose. This sensing is mediated by Gpr1 transmembrane protein and Gpa2 that physically interacts with Gpr1. When glucose binds to Gpr1, Gpa2 is activated, which then stimulates adenylate cyclase to increase cAMP production. The second component of the cAMP/PKA pathway is an intracellular system which depends on glucose uptake and phosphorylation of glucose by hexokinases, which activates the Ras proteins in yet undiscovered way. Glucose transporters are required only to maintain sufficient levels of intracellular glucose for phosphorylation and have no regulatory role in this pathway. This is also true for Snf3p and Rgt2p glucose sensors. However, as I mentioned earlier, PKA is directly involved into Snf3p/Rgt2p-Rgt1p pathway, as it phosphorylates transcriptional repressor Rgt1p. It is also interconnected with the Snf1p/Mig1p glucose repression pathway through cooperative mediation of glucose signal. Specific experiments which led to all these conclusions can be found in the following four recent reviews [73], [95], [96], [99].

In addition to the three major glucose pathways I presented so far, there is evidence that glucose transporters might be to some extent regulated by other pathways, depending on different environments. These include HXT1 induction by the high osmolarity glycerol (HOG) pathway [100], [101], HXT1-7 regulation by the target of rapamycin (TOR) pathway via Sch9 [102], [103], HXT2, 4, 5, 6, 7, 10, 11 induction by pathways involved in alkaline stress [104], HXT regulation by galactose sensing via Mth1p [105], and HXT2, 4, 5, 6, 13, 15, 16 regulation by oxygen sensing [106].

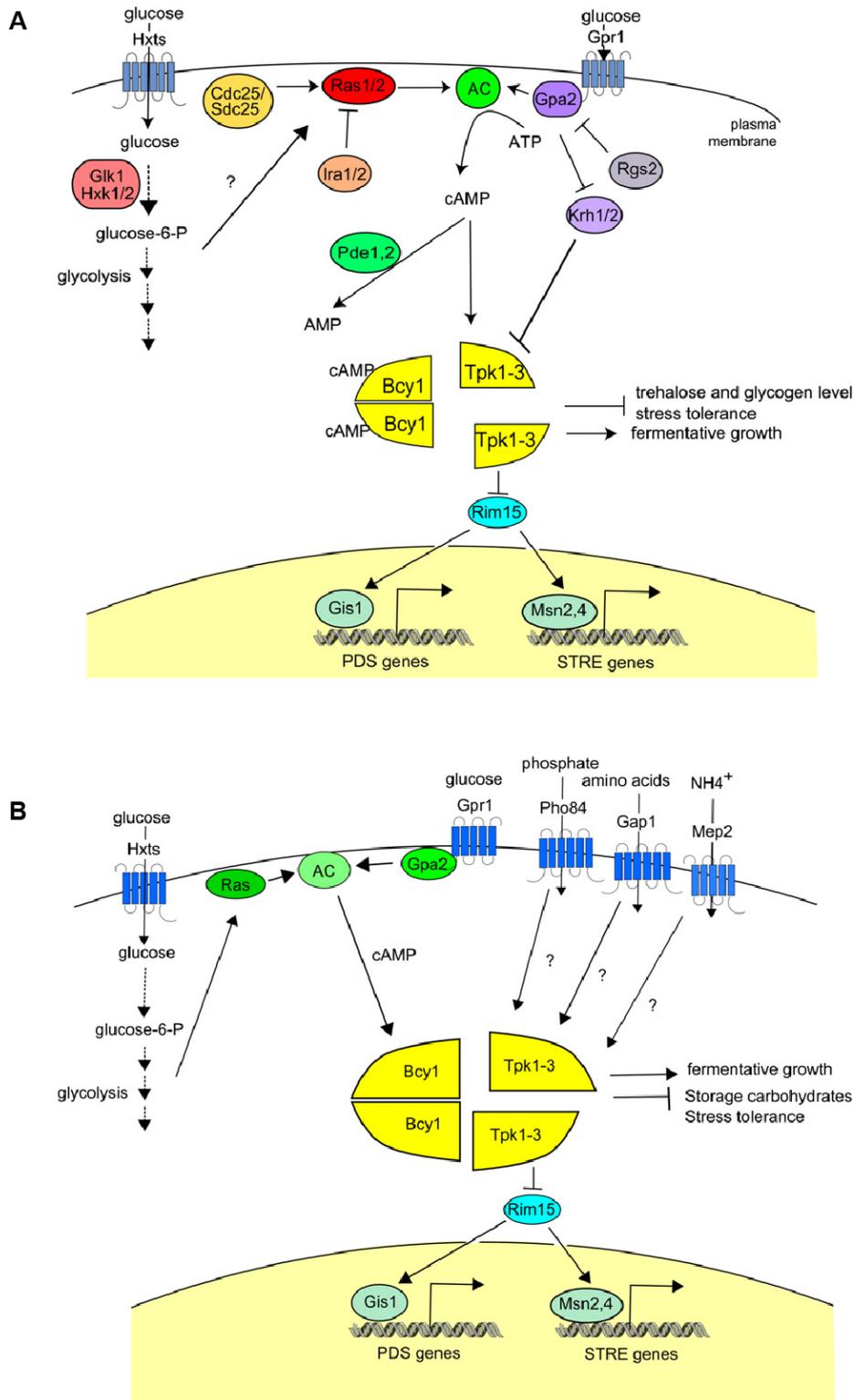


Figure 17 Schematic view of the cAMP/PKA pathway. (A) Glucose activates cAMP/PKA pathway through two branches. One senses extracellular glucose concentration via Gpr1 sensor and the other depends on the intracellular glucose metabolism. (B) cAMP/PKA pathway depends on the presence of other nutrients too. Absence of phosphates, amino acids, or nitrogen, which can be sensed by different transmembrane sensors, leads to the entry into stationary phase and low-PKA phenotype. Figure adapted from [99].

### ***A brief overview of other metabolic pathways***

Many types of nutrients are important for growth, survival, and homeostasis for yeast. Consequently, they have evolved specific pathways for different nutrients which are based on similar sensing and signalling principles already presented here. Certainly, one of them is the well-studied galactose pathway which is induced in presence of galactose. Gal2p is a galactose transporter through which galactose is taken up and then converted to glucose-6-phosphate, an intermediate of glycolysis. Signalling pathway includes a set of GAL genes and it is under control of Snf1p/Mig1p pathway, meaning that the presence of glucose will repress the expression of GAL genes. Nitrogen is another important compound needed for growth. Yeast can recognize which type of nitrogen source is in the extracellular environment and its concentration. It adjusts transcriptional, metabolic, and biosynthetic pathways depending on this perception. They also have a hierarchy of preference for different nitrogen sources, where glutamine and ammonia are the preferred ones for the lab strains. Accordingly, they exhibit nitrogen catabolite repression in which, similar to glucose repression, the preferred nitrogen sources inhibit the expression of transporters and enzymes involved in the utilization of the less preferred nitrogen sources. If nitrogen is limiting in the environment cells slow down their growth, which leads to growth arrest if nitrogen is depleted. The most prominent nitrogen related pathway is the evolutionary highly conserved TORC1 pathway which most likely responds to the intracellular amino acid levels. Amino acids can be synthesized by the cells, but they can be also imported from the environment. Cells can use them as nitrogen source and even as carbon source. Consequently, there are several different amino acid transporters which are either specific for certain amino acids or nonspecific general transporters. When yeast are starved for amino acids they trigger the general amino acid control (GAAC) pathway. This induces the global inhibition of translation initiation and more than 50 genes are upregulated, involved in amino acid biosynthesis, nitrogen utilization, signalling, and gene expression. Phosphates are also essential nutrients. Cells have evolved a PHO pathway which includes a set of PHO genes that transport and regulate phosphate utilization in dependency on the extracellular phosphate concentrations, but also intracellular needs. Yeast can also store nutrients, like glucose in the form of trehalose and glycogen. They can even recycle nutrients through a process called autophagy. The process is more complex, but to put it simply, when cells are starving on nutrients like glucose, nitrogen, amino acids, or phosphates, they can turn on autophagy which utilizes complex molecules already present in cells, degrades them, and uses them as a source of the essential nutrient building blocks. There are

many more known or less known pathways, like pathways that are induced when environmental stress poses a challenge to the survival of cells, but in my work, I focused the most on the carbon metabolism and, where appropriate, I will give more background on the processes we observed in our research. For more information about the mentioned pathways and specific experiments which led to their discovery and conclusions on their function, the reader should refer to the following reviews: [9], [32], [33], [36].

### ***Conclusion***

Reading conclusions and perspectives of the beforementioned four reviews on nutrient sensing, signalling and control of growth, recently published by groups who worked on these topics for more than two decades, one can extract some interesting and overlapping points. The first one is that nutrient sensing, signalling, and transport, and their consequence on growth is an extremely complex topic as it includes hundreds of actors, from genes to proteins, metabolites and dynamic environments, which mutually cross talk and directly interact with each other, which forms an endless network of possible interactions and cross-influences. The second point is that precisely because of this complexity we still lack knowledge and insight into some very basic questions, both specific ones about different actors and interactions and also global questions that would integrate all this knowledge. We still lack a conclusive answer to the question – “how is cell growth controlled in response to nutrients?”. We cannot really discern what is a cause and what is a consequence of growth control. Does the limited supply of nutrients results in the limited metabolic capacity which consequently affects the growth or do nutrient levels signal to the cell to rapidly adapt the biosynthetic, metabolic, and transcriptional program for the perceived levels of nutrients [73]? This has also been formalized by Barkai’s group through the theory of feedback and feed-forward strategy [107]. In a feedback strategy, gene expression is directly modulated by growth rate, while in a feed-forward strategy gene expression is modulated by environment first and then growth rate is set accordingly. The advantage of the first strategy is the robustness of the response as it depends only on the growth rate and not on the exact cause of such growth rate. The advantage of the feed-forward strategy is the ability to quickly respond and predict how the environment will affect cell physiology at later times. In this case, when a certain nutrient becomes limiting

in the environment, the cells can adapt and change their gene expression even before intracellular supply of nutrient becomes limiting to growth, and thus better deal with this limitation. Although authors support the feed-forward strategy in yeast, this question still remains unresolved or universally accepted. The third point is that we need to conduct the experiments in a context that is closer to the natural environment. Cells rarely live in perfectly homogeneous environments as we usually observe them in the laboratory. Natural environment is quite dynamic, with periods of starvation and periods of good availability of nutrients. Moreover, as cells tend to make colonies, they interact with each other and form their own microenvironments. Bringing cells closer to their natural environment will help us to better understand the unresolved question of how have evolutionary pressures shaped the growth capacity of yeast cells. The fourth point is that we often do experiments by using gene deletion, gene overexpression, gene engineering, or small-molecule inhibitors that completely inactivate the target protein. It raises the question whether the measurements we make on such modified organisms are physiologically relevant as it introduces perturbations in such a highly interconnected system. The fifth point is a hopeful one. With development of new technologies and methods in systems biology, we are able to quantify relationships between different components in cells and their environment better than ever before. This will ensure a better understanding of sensing, signalling and transport systems in the future, and their relationship to growth and adaptation across different environments.



# Growth and growth models

## Monod's growth model

I set off on a task to write a comprehensive overview of the importance of growth in microbial research and research in life sciences in general. It quickly proved to be futile. As Jacques Monod said in 1949 in his seminal paper “The growth of bacterial cultures” [108]: *“It would be a foolish enterprise, and doomed to failure, to attempt reviewing briefly a “subject” which covers actually our whole discipline”*. Monod is right in his paper which is, by the way, even today behind a paywall, a sad fact that he would probably depict as a “foolish enterprise” too. Growth is not a subject or a branch of research, it is a prevalent method, an omnipresent measurement in so many papers in microbiology and life sciences. Whatever perturbation we do to a cell or its environment, whenever we want to predict or measure an evolutionary success of bacteria and yeast, we will have to measure growth [109]. Cell growth is a quantifiable output of cells that integrates all the interactions within cells, like transcription, translation, intracellular signalling and metabolism, and all the interactions between cells and their environment which lead to assessment and adaptation to the environment by the intracellular machinery in a feedback loop that keeps proper regulation and homeostasis of cells [110]–[113]. The very basis of life is growth and propagation, so it comes as no surprise that growth is an extremely complex process which is a consequence of all genotypic, phenotypic and environmental interconnected interactions limited only by the laws of physics.

Monod rightly warns that *“The fallacy of considering certain naive mechanistic schemes as appropriate interpretations of unknown, complex phenomena should be avoided”* and he agrees that growth testifies to *“the immense complexity of the phenomena”*. However, he does think that growth follows simple laws which make it easy to define three quantitative characteristics of growth: 1) total growth, 2) exponential growth rate, and 3) growth lag. He also defines six different growth phases (Figure 18):

- I. a lag phase when growth rate null
- II. an acceleration phase when growth rate increases
- III. an exponential phase when growth rate is constant
- IV. a retardation phase when growth rate decreases
- V. a stationary phase when growth rate is null

VI. a phase of decline when growth rate is negative

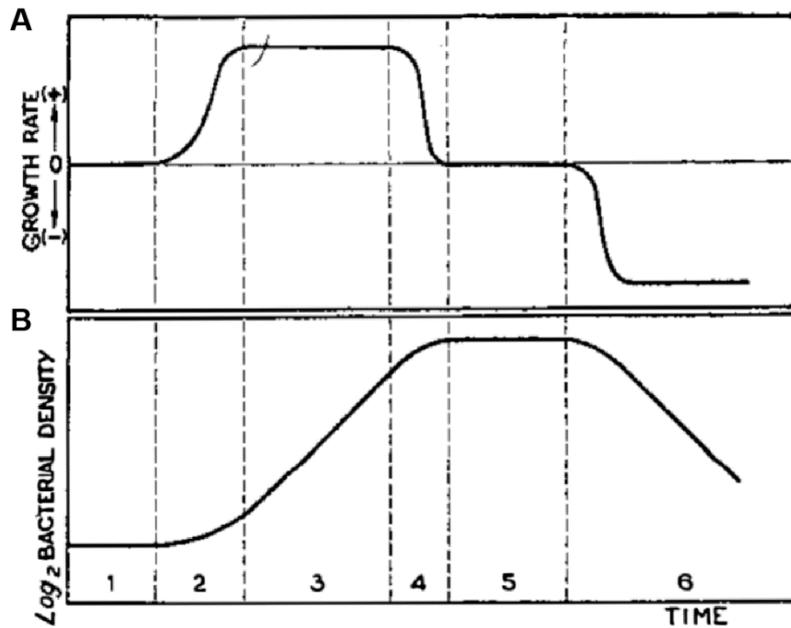


Figure 18 Phases of growth of microbial population from Monod's original paper [108]. (A) Change in growth rate defined as the increase, decrease or a constant of the number of cells in a population. (B) Bacterial optical density is linked to the number of cells in a liquid culture. Dotted lines show transitions between growth phases.

In his paper Monod proposed a mathematical model of microbial growth which is still widely used. It is a model that is very similar to Michaelis-Menten equation for enzyme kinetics. It relates the specific growth rate ( $\mu$ ) to the substrate concentration (S).

$$\mu = \mu_{max} \frac{S}{K_S + S} \quad \text{Eq. 1}$$

In which:

$\mu$  - the specific growth rate of a microorganism

$\mu_{max}$  - the maximum specific growth rate of a microorganism

S - the concentration of the limiting substrate for growth

and  $K_S$  – affinity for the substrate which is defined as the value of S when  $\mu/\mu_{max} = 0.5$  (Figure 19)

$K_S$  and  $\mu_{max}$  have to be experimentally determined in the lab which makes it empirical, unlike the Michaelis-Menten equation which was derived from the

theoretical considerations. Each  $\mu$  can be determined for a specific starting  $S$ . These empirically derived parameters differ across different species, conditions, and environments. As we increase the concentration of nutrients, growth rate of cells in a culture increases up to a point ( $\mu_{\max}$ ) where it saturates. The increase of the concentration of nutrients doesn't affect the growth rate anymore, which fits well with Monod's law (Figure 19).

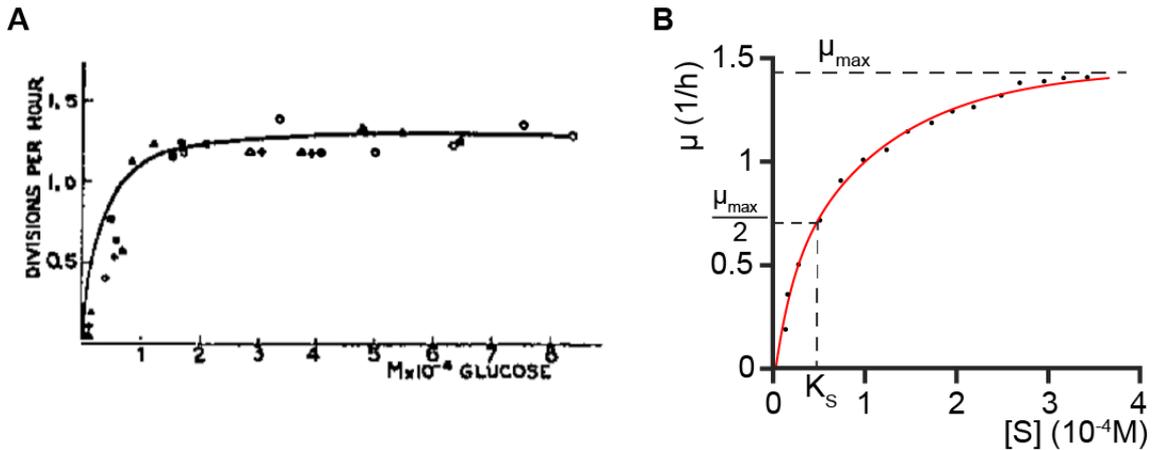


Figure 19 Division and growth of bacteria *E. coli* measured as the rate of increase of population density in liquid cultures. (A) Growth rate of *E. coli* as a function of [glucose] from Monod's original paper [108]. (B) Growth rate as a function of substrate concentration and parameters of Monod's equation.

It is worth noting here that “divisions per hour” ( $1/T_d$ , where  $T_d$  is time of division or doubling time) is not the same measurement as the growth rate ( $\mu$ ). The growth rate is the rate of population change in time. In homogeneous liquid cultures (when we look at populations), during the exponential phase cells periodically double in time, and thus the growth of population is exponential and it depends on starting population concentration ( $N_0$ ):

$$\frac{dN}{dt} = \mu N \Rightarrow N(t) = N_0 e^{\mu t} \quad \text{Eq. 2}$$

So, the time it takes for the population to double,  $T_d$ , is linked to the growth rate by the following equation:

$$\mu = \frac{\ln(2)}{T_d} \quad \text{Eq. 3}$$

Therefore, “division per hour” ( $1/T_d$ ), doubling time ( $T_d$ ), and growth rate ( $\mu$ ) are linked with a factor  $\ln(2)$ . Calculating a simple example when doubling time ( $T_d$ ) is 1.5 hours gives a value of “division per hour” of  $1/1.5\text{h} = 0.667\text{ h}^{-1}$ , but the growth rate is  $\ln(2)/1.5\text{h} = 0.462\text{ h}^{-1}$ . Hence, number of divisions per time unit is connected to but not the same as the growth rate. Even since the Monod’s time there seems to be a confusion, poor definition or poor usage of term like “growth rate”, “division rate”, and “number of divisions per time unit”.

## Uncertainty principle or constants? Is Monod’s model too simple?

The way cultures of microorganisms interact with their environment is much more complex than what can be captured with Monod’s model. For example, in different laboratories under different growth conditions the supposed constant  $K_s$  has been different by orders of magnitude [114], [115]. In the first case, researchers claim that the data strongly suggest that the large, almost 2000-fold, differences in  $K_s$  constants reported in the literature are caused by the use of *E. coli* cells adapted to different degrees to nutrient-limited growth conditions. They claim that it is probably not possible to describe the kinetic properties of a bacterium with a single set of kinetic “constants”. In the second case, researchers looked at growth of *E. coli* in residual glucose concentrations and measured dynamics of growth that did not fit Monod’s model, but rather there was an abrupt change in slope which they think came from a passive diffusive barrier prior to the uptake system that has Michaelis-Menten like kinetics. Although  $K_s$  relates to the affinity of the cell for the nutrient its biological interpretation is still a subject of debate [116]. Others could not fit the Michaelis-Menten like hyperbola leading to attempts to empirically improve the fit [117], [118]. Another problem is the fundamental microbiological parameter of growth yield, the amount of biomass produced per amount of consumed substrate (or generated ATP molecules). The growth yields were not constants as Monod supposed, but rather they varied with growth rates since the affinity for substrate depends on its concentration [119], [120]. In natural populations the proposed growth phases can vary independently in different conditions [121], [122]. Even today there are two schools of microbiologist which contradict each other. One school claims a positive growth rate – growth yield correlation based on assumption of constant maintenance energy (energy consumed by cells for other things than growth). The others say that there is a growth rate – growth yield trade-off due to

different selective pressures in different environments [123]. To conclude, homeostatic mechanisms, adaptation, relationships between growth affinities and nutrient transport, membrane permeability, cell energetics, effects of glucose concentrations on gene expression, effects of stationary phase gene expression on growth constants, genotype used, inoculum history, length of exposure to different glucose concentrations, dynamical changes in the environment, cell memory, cooperation, competition and other phenomena all contributed to high complexity and diversity of the growth of microorganisms. This is the reason why, both from the experimental and especially modelling point of view, elucidating growth is a challenging matter still riddled with many unknowns. I will finish with a quote from a nice review on this topic by Thomas Ferenci with which inspired this paragraph and with which I very much agree: “...just like in the quantum mechanical world where a measurement perturbs the system leading to uncertainty, a bacterium interacting with the environment perturbs that growth environment. Nutrient utilisation results in changed environmental conditions and hence changed gene expression and hence altered physiological properties and a different  $K_s$  property. Further growth leads to further adaptation and...uncertainty.” [124]. This is also true for very common cases, probably the most fundamental method in genetics, when we “perturb” the genome of cells through gene deletions (and with the rise of systems and synthetic biology – construct artificial gene expression control), which leads to a great insight in the function of genes or gene networks but also perturbs the physiology of the cell.

## Hallmarks of yeast growth in liquid environment

Yeast *Saccharomyces cerevisiae*, very similarly to bacteria *Escherichia coli*, exhibits the growth phases as described by Monod. Scientists usually grow yeast in liquid batch cultures and continuous cultures like chemostat. In chemostat fresh medium is constantly added at the same time as the metabolic products, microorganisms, and leftover nutrients are constantly removed in order to keep the volume of the culture constant. When cells are first inoculated into fresh media they go through a lag phase during which they adapt to new environmental conditions (Figure 20). Glucose is by far the most preferred carbon source for yeast *S. cerevisiae*.

Cells start to use glucose for their growth and enter the exponential phase of growth. While they are consuming glucose, they produce ethanol by fermentation. Exhaustion of glucose leads to growth arrest and a switch from fermentative to respiratory metabolism. Cells go through a diauxic shift, an adaptation of metabolism to be able to utilise another carbon source, ethanol. This transition is not as simple and straightforward as it is usually simplified in such schemes. There is a considerable heterogeneity of response in complex environments both between and within populations of microorganisms [125]. By doing this, cells can in a way anticipate and predict their future environments by “hedging their bets” to what may come, protecting themselves on a population level from uncertainties of dynamic and, often harsh, environments [126]. During the post-diauxic phase cells grow slower on ethanol until they exhaust it all. At this point they enter the stationary phase. This phase is characterized by major shifts in gene expression and metabolism. Cells stop to proliferate and they find themselves in a, so called, quiescent state. Transcription and translation slow down severely, accompanied by reduced expression of genes that encode ribosomal proteins and induced transcription of stress responsive genes which enhance resistance to stress, cell start to accumulate and store carbohydrates, cell wall becomes thicker, chromosomes condense and autophagy kicks in during which cells degrade and recycle their cellular components [127]. After that, if no fresh nutrients are provided and culture is not diluted, cells age and die.

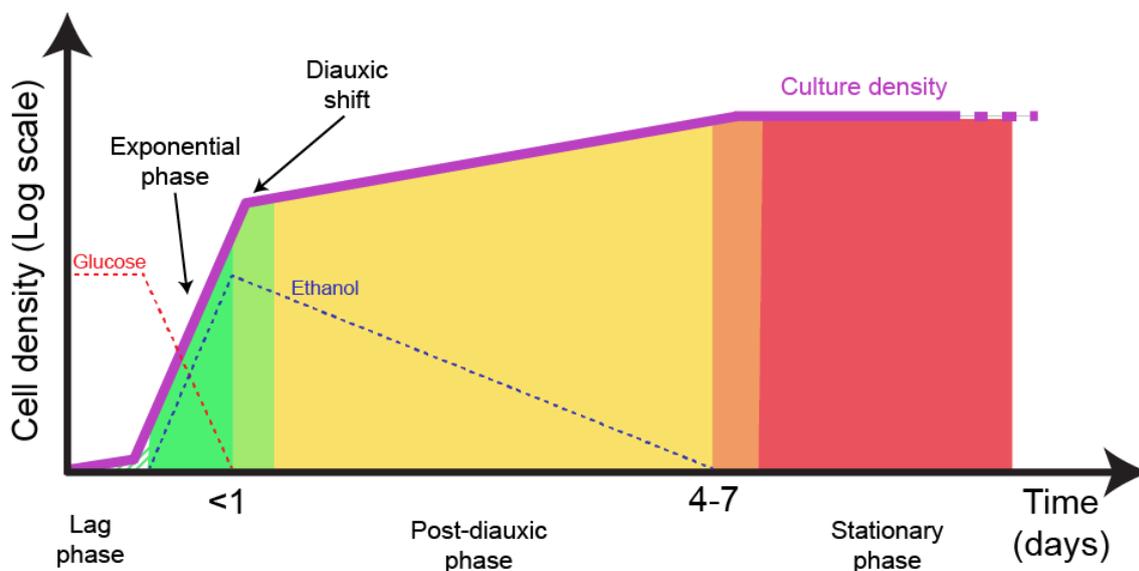


Figure 20 Phases of growth of yeast *Saccharomyces cerevisiae*. Figure adapted from [95]. Inoculated cells go through a lag phase (green lines) while they adapt to new environment. They enter an exponential phase of growth (green), rapidly consume glucose (red dashes) and produce ethanol

(blue dashes). Once glucose is consumed, cells go through diauxic shift to switch to consumption of ethanol (green to yellow transition). In post-diauxic phase (yellow) cells grow slower on ethanol as their carbon source. When they deplete all the ethanol, cells go through major metabolic switch and enter a quiescent stationary phase of no growth (red).

There are many factors that affect yeast growth (Figure 21). Glucose or any other nutrient is sensed by the cells and transported through transporters on the cell membrane. These nutrients are processed by the cells and used for growth, cellular reserves, maintenance, and some are excreted from the cell to the environment. During this process cells constantly change their environment either by taking up nutrients from the environment and by excreting metabolites which can be used for signalling and growth, while some of them can inhibit and harm the cells too. The environment can be harsh so cells evolved many mechanisms and adaptation strategies to different stress types like nutrient depletion, osmotic stress, heat shock, salt stress, and oxidative stress [128]. All the environmental factors have to be integrated into the constant adaptation and response by cells in an everchanging environment. Until they die. Even then they burst and release their cellular content that changes the environment and permits other cells to scavenge on them.

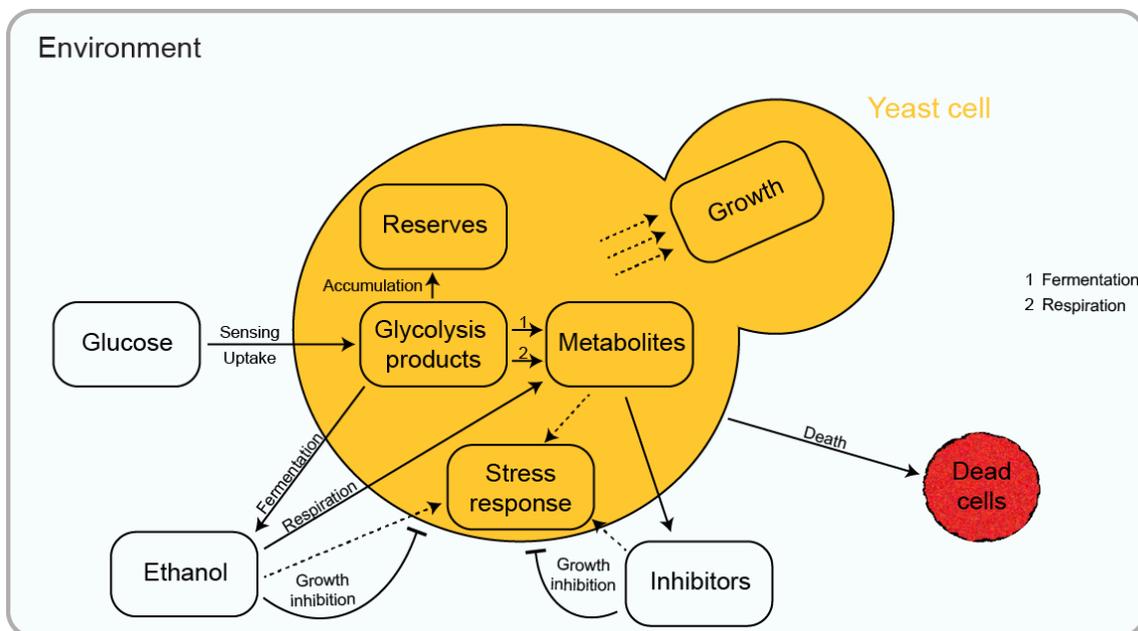


Figure 21 Simplified diagram of yeast metabolism and growth. Figure adapted from [129].

## **Yeast colonies**

### **Models of colony expansion**

Until now, we considered growth in batch or continuous cultures which mostly consist of homogenous environments – all cells in a population find themselves surrounded by the same concentration of the same molecules. Most of research is done in such a way because it removes the variation between cells that would otherwise stem from heterogeneous environments. We discovered many exciting phenomena doing so, nevertheless cells in the natural environment often form colonies or biofilms, which are spatially structured formations that form heterogeneous environment within them. The physico-chemical environment inside colonies is heterogeneous and as such, cell at different positions will find themselves in a different environment. This makes it difficult to calculate important biological parameters like population growth and yield. What governs growth in a colony is the fact that different cells have access to different nutrients, therefore depending on their positions, some cells grow faster, some slower, and some do not grow at all. To predict a colony expansion rate, we would need to know the number of cells ( $N$ ) that are growing in a colony and at which rate. This is not a trivial matter. Nutrients diffuse from the environment into the colony, nutrients and metabolic products diffuse inside the colony, and cells uptake nutrients from their local environment. All of these phenomena produce complex spatio-temporal variation of the microenvironment inside a colony. In turn, this leads to a spatial phenotypic variation. Even when we study colonies in the laboratory, we often grow them on solid agar gel which contain a finite amount of nutrient. Cells divide on agar, they push each other as they grow and expand the borders of their colony. In the centre, cells consume and progressively deplete the nutrients in the gel, while cells that are pushed on the edges of a colony constantly invade areas where nutrients are not depleted yet. This makes colony growth predictions complex and hard to reach.

We can roughly divide colony growth into the following phases (Figure 22):

- I. Lag phase after inoculation of cells during which cells adapt to new media.
- II. Exponential growth phase during which there is sufficient amount of nutrients delivered to the whole colony through diffusion.
- III. Deceleration phase as colony grows bigger and conditions become restricting for growth of the whole colony.

- IV. Linear growth phase during which radius of the colony grows linear in time because only cell at the front have access to nutrients and cells at the middle of the colony deplete their nutrients [130]–[132].
- V. Death phase if nutrients from the gel are completely depleted (or cells simply reach the border of a petri dish). In principle, linear growth phase should be constant in an infinitely big petri dish with homogenously distributed nutrients.

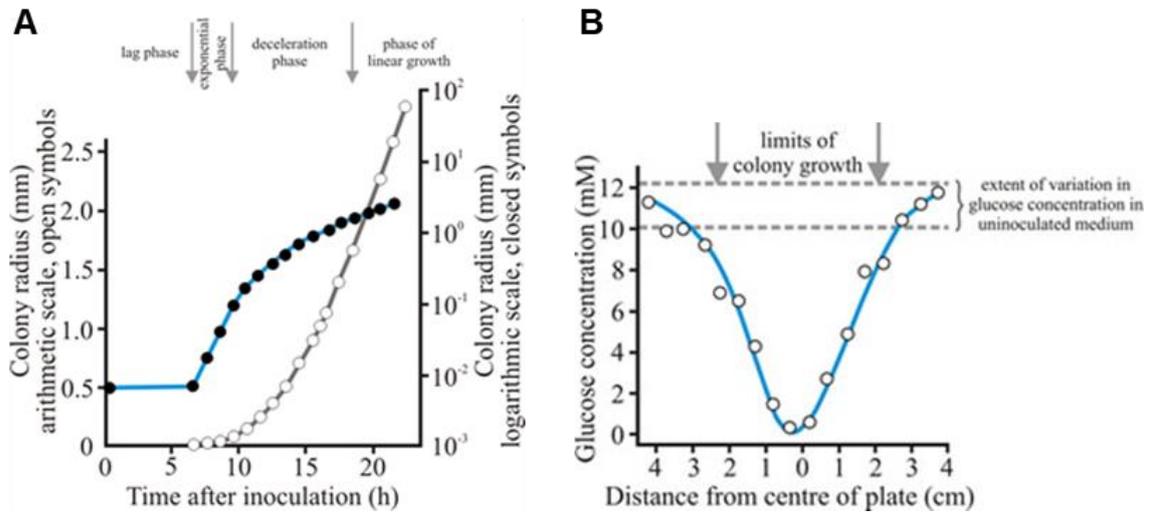


Figure 22 Growth phases of yeast colony and glucose depletion in gel. Figure adapted from [133]. (A) Growth phases of *Aspergillus nidulans* colonies. (B) Glucose concentration in the medium below a colony of *Rhizoctonia cerealis* grown at 25°C on 20 ml of agar medium in a 9 cm Petri dish.

Fifty years ago, in 1967, Pirt suggested a model of microbial colony growth [131] that accounts for the changes of the expansion rate with time. He observed that after about 12 hours of growth colonies start to radially grow at close to constant rate. The model assumed that there is a constant number of cells at the colony front which expand into steady nutrient concentration in gel (Figure 23). Cells constantly uptake nutrients from gel meaning that in the area where cells stay longer, like in the middle of the colony where cells were inoculated, nutrients will be depleted at one point and cells will enter into a growth arrest. He proposed the following equation for colony radial growth rate:

$$\frac{dr}{dt} = k_1(\sqrt{C_0} - \sqrt{C_i})\sqrt{\mu_{max}} \quad \text{Eq. 4}$$

The parameters are:  $r$  - colony radius;  $k_1$  - experimentally derived constants;  $C_0$  - initial glucose concentration;  $C_i$  - minimal glucose concentration that need to be exceeded before cells can start to grow;  $\mu_{max}$  - maximal cell growth rate.

$C_i$  is usually very small or negligible. So, growth of colony radius will increase in time with the increase in starting glucose concentration, maximal cell growth rate, and experimentally derived constant  $k_1$  which varies between different microorganisms.

A couple of years later, Gray and Kirwan expanded this model to include a constant  $k'$  [130]. The idea was to take into account that cells at the edge of the colony consume nutrients for growth given as the metabolic yield ( $Y$ ) and that these nutrients need to diffuse through the medium into the colony.

$$k' = k_2 \sqrt{YD} \quad \text{Eq. 5}$$

$$\frac{dr}{dt} = k_2 \sqrt{YD} (\sqrt{C_0} - \sqrt{C_i}) \sqrt{\mu_{max}} \quad \text{Eq. 6}$$

The newly introduced parameters are metabolic yield of the colony ( $Y$ ), diffusion coefficient of glucose through the medium ( $D$ ), and dimensionless growth velocity  $k_2$  which is again an empirical constant that depends on diffusion coefficient, nutrient concentration, and growth rate. They define the metabolic yield as number of cells produced per unit mass of glucose consumed under condition of two-dimensional colony with uniform thickness. This is similar to the yield in liquid cultures which is defined as  $Y = \mu/q$ , where  $\mu$  is the growth rate of cells and  $q$  is the uptake rate of the nutrient used for growth.

## Colonies form spatial structures

It is interesting to see that nutrient level in microbial colonies affects spatio-genetic structuring and diversity in microbial colonies [134]. As Mitri and her colleagues show, as long as there is a limited number of cells growing at the colony edge, genetic drift will be promoted (Figure 23). Increase in colony expansion velocity, which increases with nutrient concentration, leads to decrease in the rate of diversity loss. Said differently, higher nutrient limitation promotes genetic demixing distance closer to the starting well mixed inoculum. Demixing is caused by genetic drift and fitness difference where cells of a certain genotype randomly overtake the local population at the expanding frontier. Given enough space for expansion, cells of a certain genotype will eventually separate in space within colonies. This happens both in yeast and bacteria.

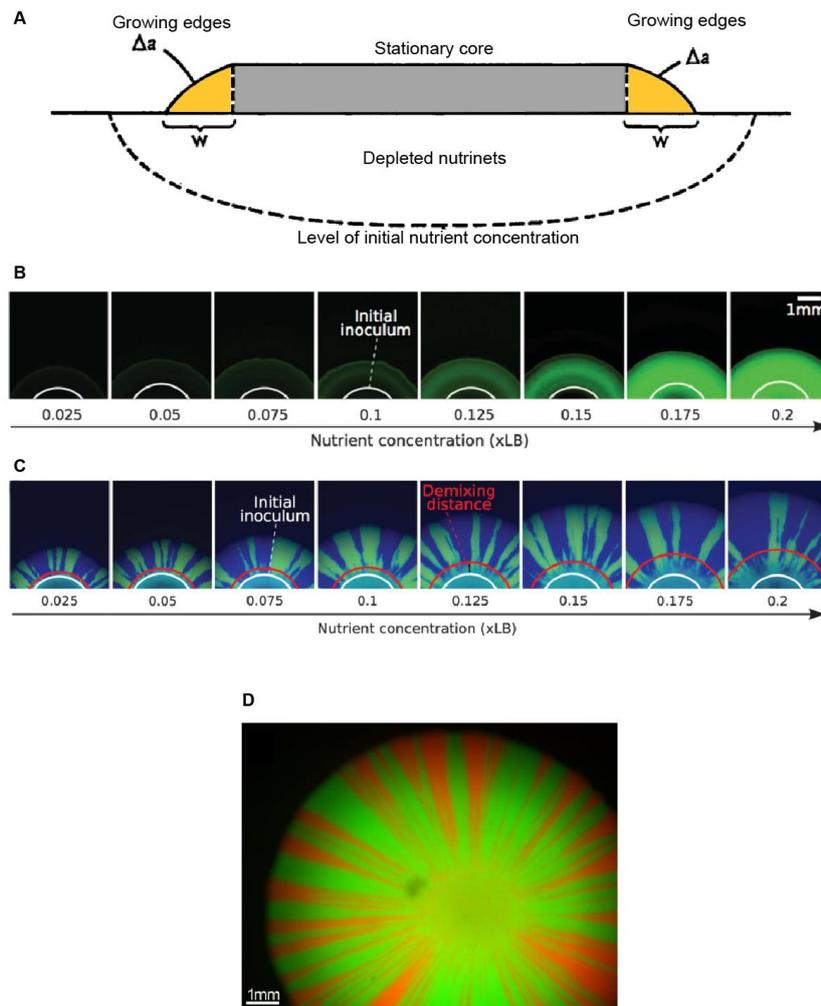


Figure 23 Colony growth and genetic drift. Figure adapted from [131], [134], [135]. (A) Pirt's model of colony growth. Cells grow at the edge of the colony where they have access to nutrients. Cells in the middle of the colony do not grow because nutrients are depleted beneath them [131]. (B) Green fluorescent marker is expressed in bacteria *Pseudomonas aeruginosa* in the parts of the colony that actively grow. As nutrient concentration in gel is increased, growing edges become wider [134]. (C) YFP and CFP labeled *P. aeruginosa* 1:1 mixture. Genetic demixing arises due to genetic drift and the point of genetic demixing depends on the nutrient concentration in the gel [134]. (D) Genetic demixing happens in yeast *S. cerevisiae* too. Compared to bacteria, the number of sectors is larger [135].

Concentrations of nutrients like glucose are homogenous in well mixed cultures, they depend on cell properties like nutrient uptake rate. However, in structured cell assemblies like colonies, concentrations depend on spatial organization too. Meaning that nutrients diffuse into colonies and cells uptake them from their environment according to a model I will present here.

Molecules, like nutrients necessary for growth, tend to diffuse from regions of high concentrations to regions of low concentrations of said molecules. This means that there is a flux of nutrients diffusing from the nutrient source through the colony. To understand this we need to go back more than a century and a half at a time when Fick published his paper “Ueber Diffusion” (“On Diffusion”), where he described the diffusive flux known now as the first Fick’s law and how diffusion causes a change in concentration in time known now as the second Fick’s law or simply diffusion equation [136]. He starts by saying something that can be generalized about the relation between biology and physics that is still pertinent today: *“Liquid diffusion through membranes is not only to be regarded as one of the elementary factors of organic life, but also as a highly interesting physical process, which is far more important to physicists [and biologists; n.b. Z.M.] than has hitherto been considered... The reason for this sparse treatment is partly due to the great difficulty of making precise quantitative experiments in this field. And, in fact, it is so great that I have not yet succeeded in bringing the dispute between the theories to a definite conclusion.”* Fast forward century and a half – we still struggle to completely understand the response of cells to different environments and how are different signalling pathways integrated and regulated to give rise to nutrient uptake properties through membrane transporters. Although in his specific case he was proven correct, like in this example from 2014 when Patzek revisited Fick’s experiments and concluded: *“Fick’s experimental approach was sound and measurements were accurate despite his own claims to the contrary”* [137].

The first Fick’s law states that the flux per unit area or “flux density” of a chemical is proportional to its concentration gradient:

$$J = -D\nabla C \quad \text{Eq. 7}$$

$J$  is the diffusion flux: the amount of nutrients that will flow through a unit area during a unit time interval.  $D$  is the diffusion coefficient in the units of area per time ( $\text{m}^2 \text{s}^{-1}$ ). It is proportional to the squared velocity of the diffusing particles and it depends on temperature, viscosity, and size of the particles.  $C$  is the concentration of a nutrient of interest.  $\nabla$  (nabla) is the gradient mathematical operator. The negative sign shows that diffusion occurs from higher to lower concentrations.

Diffusion fluxes cause a change in the distribution of concentrations of nutrients, so from this Fick derived a differential equation for diffusion known as the second Fick’s law:

$$\frac{\partial C}{\partial t} = D\Delta C \quad \text{Eq. 8}$$

Here we introduce a second order differential equation known as Laplace's equation.  $\Delta$  (delta) is the Laplace operator.

Cells are not as simple as one diffusion equation. When they grow as a colony they form a three-dimensional structure in space through which nutrients diffuse, but they also consume those nutrient and secrete different metabolites, among other things. If we take glucose as an example, cells also express transporters on their membranes to uptake glucose and effectively act as a sink of nutrients. For three-dimensional cell assemblies, we can calculate the amount of nutrients present at each point of the colony as follows:

$$\frac{\partial C}{\partial t} = D\Delta C - q(C) \quad \text{Eq. 9}$$

Concentration of nutrients changes in time and depends on the diffusion coefficient ( $D$ ), local concentration gradients, and the uptake rate of nutrients ( $q$ ) which is the amount of nutrients cells uptake per unit time and unit volume of a colony. It may seem simple, but this equation is not easy to solve since it will depend on the colony morphology, boundary conditions, and the uptake rate of nutrients which actually changes with the nutrient concentration. To illustrate the complexity, yeast *S. cerevisiae* has 20 presumed glucose transporters each differentially expressed at specific environmental conditions, including different glucose concentrations, and regulated through three major signalling pathways whose mechanisms are not completely elucidated [138]. Therefore, the problem is not to solve the diffusion equation which can be done numerically, but to understand how uptake rate depends on the glucose concentration.

This model can be simplified in some cases like infinite planar biofilms or cell assemblies made out of one layer of cells only because parameters evolve in one dimension (or quasi one dimension) only. Meaning that variation of nutrient concentrations and growth rate need to be solved for one dimension only. Then the equation looks like this:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} - q(C) \quad \text{Eq. 10}$$

## Models of growth limitations caused by toxic metabolites

As I mentioned earlier, cells produce metabolites that can have some inhibitory effects on cell growth. I will briefly mention two attempts of modelling those effects coupled with growth. First one is a model of growth of *Bacillus subtilis* biofilms. They reproduced velocity field of the biomass across the biofilm and predicted that fluctuations in the growth rates of the bacteria lead to roughening of the growing bacterial layer [139]. To explain these surface heterogeneities as well as inhibition of growth rates, they introduce the production of toxic by-products:

$$\frac{\partial \rho}{\partial t} = D_{\rho} \Delta \rho + P_{\rho} c \quad \text{Eq. 11}$$

Here,  $\rho$  (rho) is the concentration of toxic by-products,  $D_{\rho}$  is diffusion of toxic by-products,  $\Delta$  is Laplace operator,  $P_{\rho}$  is the rate of production of toxic by-products which they, for simplicity, put as a constant, and  $c$  is the cell density.

As growth is limited by waste, they use the following model to link the dependence of growth rate on the presence of toxic by-products:

$$\mu(\rho) = \frac{\mu_{\max}}{1 + \left(\frac{\rho}{\rho_i}\right)^m} \quad \text{Eq. 12}$$

Here,  $\mu$  is the growth rate,  $\mu_{\max}$  is the maximal growth rate,  $\rho_i$  is a typical inhibitory value for  $\rho$  (the growth reaches half maximum when  $\rho = \rho_i$ ),  $m$  characterizes how steeply growth is inhibited.

Through experiments they concluded that shifts in pH were responsible for growth inhibition. The only compound that accumulated at sufficient levels to change the pH was ammonia. They followed the growth of a biofilm at different positions of a biofilm measured as the distance from centre of a biofilm and saw that growth is the fastest at the edges, with expansion velocities reaching  $250 \mu\text{m h}^{-1}$ , and the slowest close to the centre. Growth at the edges reached the maximum at 8h after inoculation and then it monotonically decreased over time, in part due to accumulation of ammonia. The growth dynamic was captured well with their model.

In the second inhibition related model researchers looked at growth of yeast *Pichia membranaefaciens* [140]. Cells grew on an agarose gel, but the setup was

designed in such a way that the media in the agarose gel was constantly replenished. Change in the thickness of the agarose gel led to a difference in colony morphology at the front. This is their model:

$$\frac{dC_m(x,t)}{dt} = D\Delta C_m(x,t) - \frac{1}{\tau}C_m(x,t) + \frac{1}{h}\theta(w(x,t)) \quad \text{Eq. 13}$$

In this equation  $C_m$  is the concentration of metabolites,  $h$  is the thickness of the gel,  $\tau$  (tau) is the characteristic time for metabolites to disappear through the agarose gel ( $\tau \approx h^2/D$ ), and  $\theta$  (theta) is the metabolite source term that depends on  $w$  which is the yeast field.

When they couple the change of concentration of toxic metabolites to growth, this model results in a local accumulation of metabolites at the edge of the colony with effects on growth inhibition and colony morphology. This model also quantitatively agreed well with the experimental observations.

There are many microorganisms that are motile, they have flagella or other mechanisms which help them propel through the environment. Cell movement is yet another important factor of the colony growth which leads to a wide variety of pattern formations and colony morphologies. In some cases, they are so beautiful that it gave rise to a whole artform called “bacterial art” [141]. Ben-Jacob was a pioneer in the study of bacterial intelligence, self-organization and social behaviours of bacteria and an influential figure in the establishment of the physics of living systems field (Figure 24). His group, as well as some other groups developed an extensive theoretical and experimental framework to study motile bacteria and how they adapt to different environments [142]–[146]. Cell movement is a very important evolutionary trait that cells developed, because they can move through the environment to get away from adverse environments or to find nutrients for growth. They looked at colonies on agar plates that can branch and form fractal-like patterns. In such models, bacteria consume nutrients from their environment but can also move through “bacterial diffusion” in the environment and show higher level social behaviour. These models are often dimensionless, so the biological function remains elusive, even though they capture the pattern formation well.

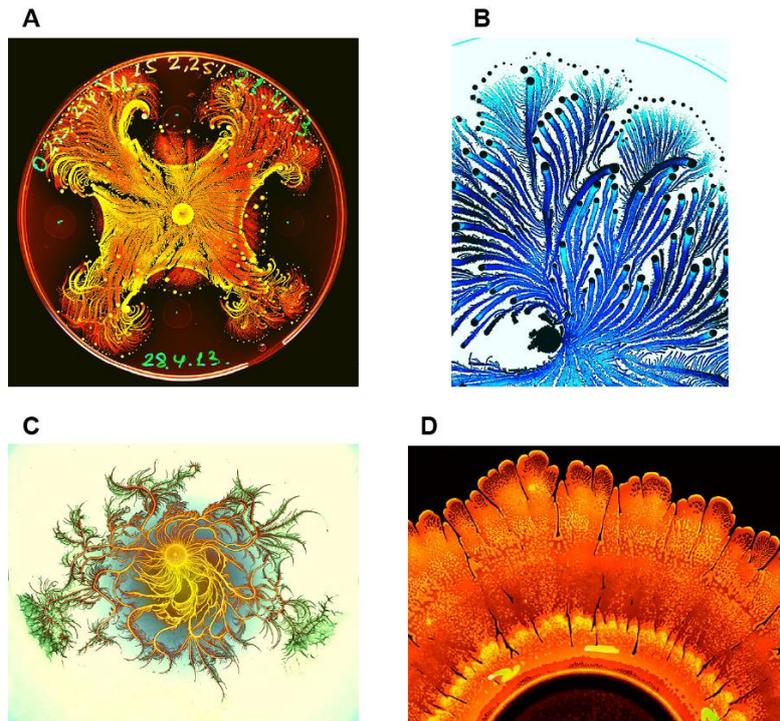


Figure 24 Complex morphologies of bacteria that evolved motility and social behaviors, discovered in Ben-Jacobs's laboratory and used as "bacterial art". (A) *Paenibacillus vortex* exposed to a chemotherapy substance. (B) Vortex Blue (*P. vortex*). (C) Bacterial Dragon (*Paenibacillus dendritiformis*). (D) A close look at *P. dendritiformis*. Figure adapted from [141].

## Long-range metabolic dependence in colonies

Growth of colonies is not always continuously linear in time even though nutrients needed for growth are present. In a fairly recent discovery Süel's group discovered that *B. subtilis* grows in a periodic fashion [147]. The population of cells oscillates between a period of growth and a period of growth arrest (Figure 25). They used a microfluidic device that can trap cells in the middle of the device while being surrounded with a constant flow of media. This gives rise to a circular population of cells, similar to a colony on a petri dish, only significantly thinner. There is a competition for nutrients between cells in the periphery which have a direct access and cells in the interior which get much less or no nutrients due to diffusion and uptake by the cells in the periphery. But there is also a conflict that arises. Cells in the periphery protect cells in the interior from adverse external shocks. They are the first line of defence from which the cells in interior benefit. In order to benefit from peripheral cells during adverse times, they need to sacrifice the lack of nutrients when environmental conditions are mild and support growth of the cells in the periphery. In this particular case *B. subtilis* seems to evolve a very

slick mechanism. After a certain colony size is reached (diameter of  $580 \pm 85 \mu\text{m}$ ), oscillations in growth emerge. Cells in the periphery stop to grow for a period of time during which cells in the interior can get nutrients needed to keep them alive. These oscillations continue in time. They describe this phenomenon as emergence of long-range metabolic co-dependence between peripheral and interior cells. Metabolic dynamics of every cell in a population can be regulated in the context of the community, although bacteria are single cell organisms. They determined that carbon source did not have any role in oscillations, but rather the oscillations appeared due to nitrogen limitation. There are three key players in this: glutamate, glutamine, and ammonium. Glutamate is usually supplemented in the growth medium. Cells uptake glutamate and together with ammonium process into glutamine which is essential for growth. Glutamate is the most abundant in the periphery because it is in the media and cells in the interior get only limited amounts of it because cells in the periphery uptake the most of it. Glutamate is needed for production of ammonium but due to specificities of ammonium production it will be more abundant in the interior. So, this is what happens according to their results: the excess glutamate not consumed by the biofilm periphery diffuses to the interior where it can be converted into ammonium. The ammonium in turn enhances growth in the periphery because of the production of growth promoting glutamine, and this consequently reduces the supply of glutamate to the interior because cells are growing at the periphery. It is a delayed negative feedback loop between glutamate consumption and ammonium production.

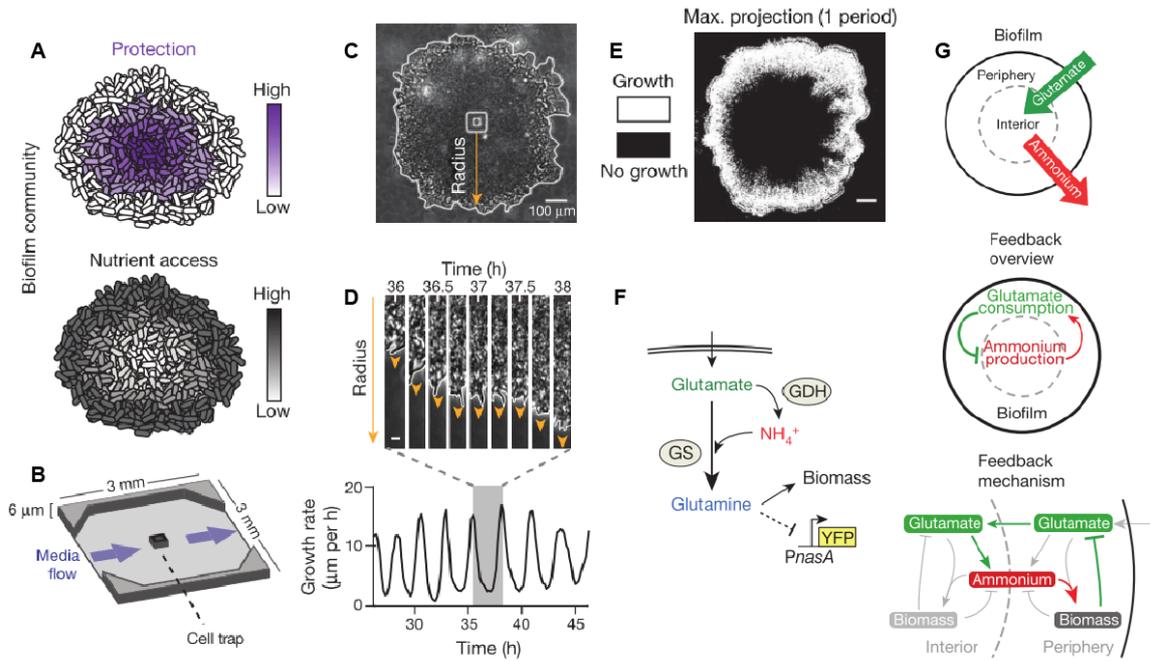


Figure 25 Oscillating growth of the same cell population. (A) Sketch of heterogeneous environment inside a colony. (B) Microfluidic device for cell growth and imaging. (C) A grown biofilm. (D) Oscillations in the expansion of the biofilm. (E) White parts of the biofilm grow, while the dark parts do not. (F) Glutamate transformation to glutamine. (G) A model of glutamate and ammonium dynamics inside a biofilm. Figure adapted from [147].

In a follow up study published this year they looked at two spatially distant populations that have access to the same nutrient flow [148]. These two populations compete for nutrients, but to overcome this problem they employ electrical cell-to-cell signalling mediated by potassium ion channels to communicate over a distance and start to oscillate in an anti-phase when resources are limited (Figure 26). Populations synchronize in such a way that population one grows, then population two grows, then population one grows again, and so on. They describe this as emergence of nutrient time-sharing, each population takes its turn to consume nutrients.

Both examples, intra- and inter-population growth dynamics, are a testament to astonishing diversity and complexity of growth dynamics that evolved in microorganisms, propelled both by biological mechanisms and physical laws and constraints.

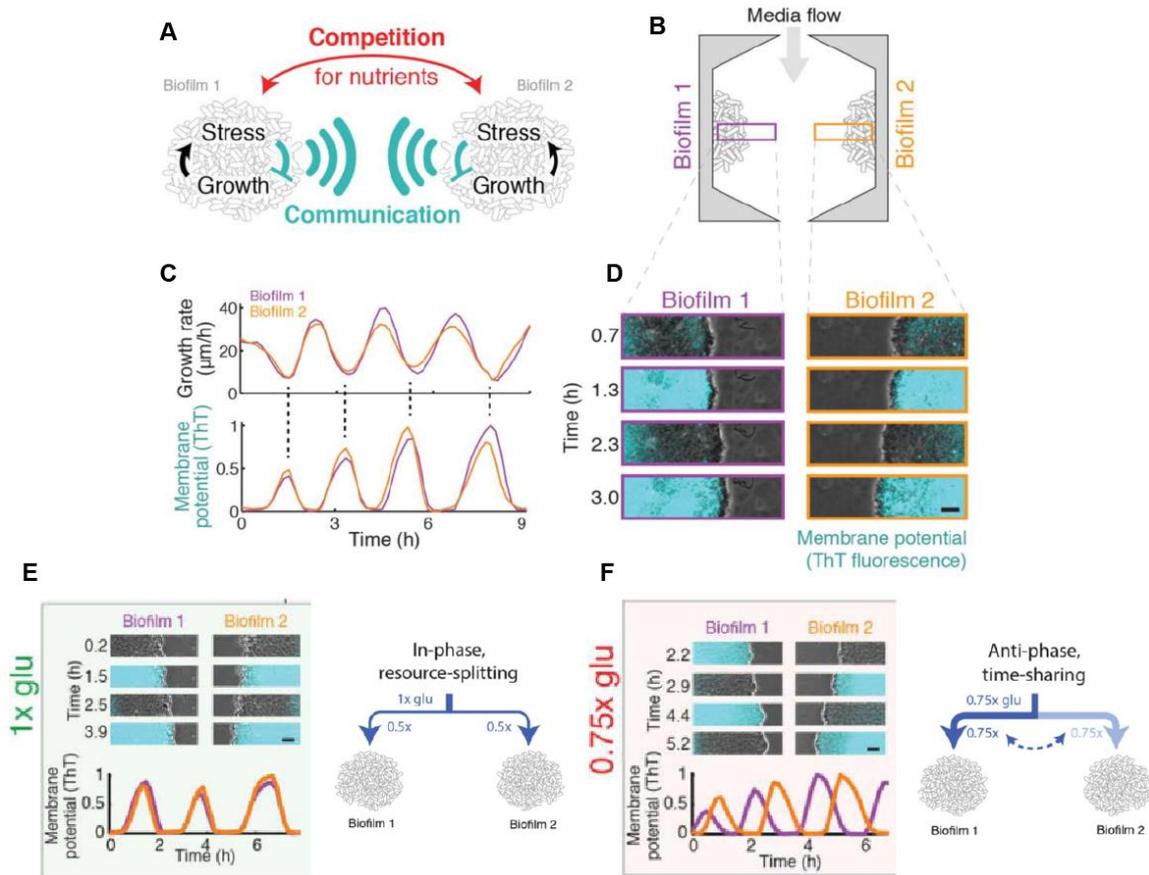


Figure 26 Oscillating growth of the two distinct cell populations. (A) A sketch of two biofilms in cooperation and competition. (B) Microfluidic device for biofilm growth. (C, D) Biofilm growth. (E) In-phase resource splitting. (F) Anti-phase time-sharing. Figure adapted from [148].

## Growth of cylindrical yeast colonies

Finally, the last example of experimental and modelling research on colony growth that I will present, and possibly the most pertinent one for my research. A couple of years ago Vulin and his colleagues were wondering how they could simplify growth of the colonies such that their observations could be better understandable and their model more straightforward [13]. They discovered that yeast *S. cerevisiae* can be grown on an agar plate in any desired shape [149]. They separated the contact between the gel and the cells with a porous membrane. Through this membrane all the nutrients can freely diffuse. In order to confine vertical cell growth, they developed a method where they put a silicon based organic polymer polydimethylsiloxane (PDMS) on the parts of the membrane where they

want the pores to be blocked. Doing so, cells do not have access to nutrients in the blocked areas so cells will grow only in the shape of a pattern where nutrients freely diffuse through the membrane (Figure 27). Basically, they impose the area of nutrient uptake and eliminate the horizontal growth of colonies on the plate. Somewhat surprisingly, cells in this case grow vertically upwards. They chose a circular pattern, so yeast colony grows into a cylindrical shape. The main difference now, apart from the fact that they can choose a confined shape that is easier to model, is that cells do not expand into areas where nutrients are abundant, but rather cells divide only at the bottom of the cylinder and push cells upwards in the air. This way, uptake area (radius of the colony) is known and constant, and they can mathematically compute the flux of nutrients into the colony.

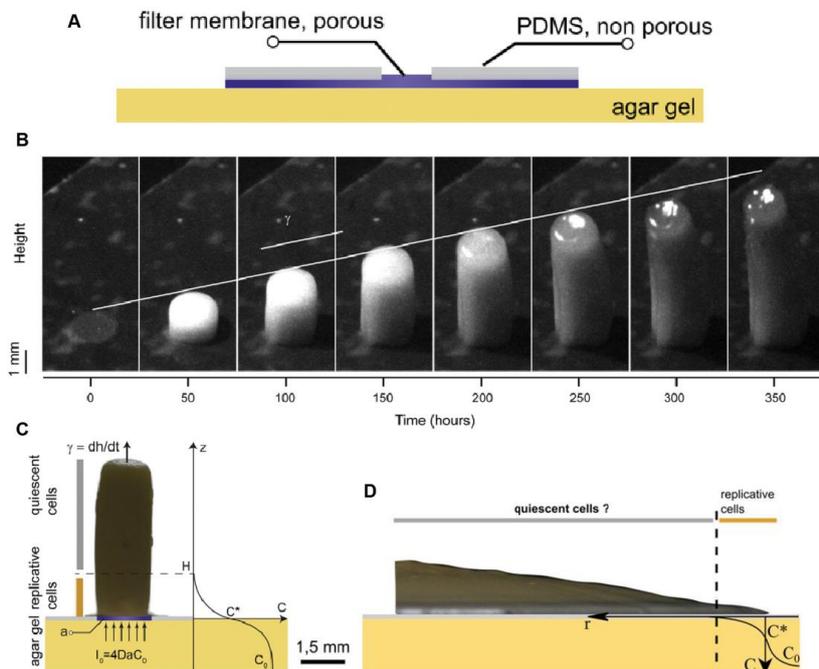


Figure 27 Growth of cylindrical yeast colonies. (A) Setup of the filter membrane on the agar gel. (B) Growth of a yeast cylinder. (C) Quiescent and replicating cells in the cylindrical colony. (D) Quiescent and replicating cells in a colony on a gel. Figure adapted from [13], [149].

They discovered that the cylindrical colony quickly reaches a steady state where its growth rate is constant. Also, the wider colonies grew faster than the thinner colonies and their growth rate was inversely proportional to their radius. At one point the colony growth rate saturated as glucose concentration was increased (Figure 28). It is possible that this happens due to the accumulation of toxic metabolites that inhibit the growth of the colony, especially in higher glucose concentration when production of metabolic by-products like ethanol is very high. By combining growth rate measurements with their glucose influx estimations

(which depends on the glucose uptake capacity), they were able to measure yield. As the glucose concentration in the gel increased, the yield of cylindrical colonies decreased. In well mixed liquid cultures growth rate of each single cell is the only parameter that defines the growth rate of the population. However, in colonies it is the colony metabolic yield that defines growth and not the single cell growth rate. The importance of the metabolic yield reflects in the interplay between glucose uptake rate and growth rate. If cells uptake less glucose, glucose can penetrate deeper in the colony that can be used for growth of more layers in the colony. However, if cells uptake more glucose, glucose will not be able to penetrate deeper in the colony and less layers will grow which will lead to a smaller growth rate of the colony. Therefore, the colony metabolic yield becomes the critical parameter in colony growth.

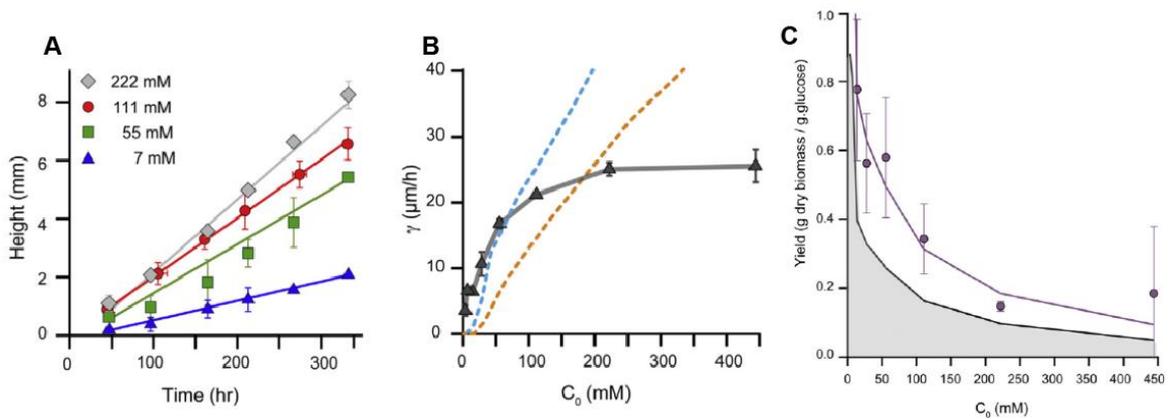


Figure 28 Growth rate and yield of cylindrical colonies. (A) Height of the cylindrical colony in different glucose concentrations. (B) The velocity of cylindrical colony expansion. (C) Cylindrical colony yield in different glucose concentrations. Figure adapted from [149].

To model their system, for approximation of steady state influx, they used the diffusion equation I mentioned before ( $\partial C/\partial t = D \Delta C$ ). Combining this with Fick's first law they got a simple solution for flux at the bottom of their colony:

$$I = 4Da(C_0 - C^*) \quad \text{Eq. 14}$$

Where  $I$  is the maximum flux that can enter inside a colony,  $D$  is diffusion coefficient,  $a$  is the radius of the colony,  $C_0$  is starting glucose concentration in the gel,  $C^*$  is glucose concentration at the interface with cells which will depend on colony uptake rate  $q$  and diffusion of glucose through the gel.

Assuming that diffusion in the colony happens in only one dimension, they used the model of concentration change in the colonies which includes uptake rate ( $q$ ). Under the assumption of a steady state, solving one dimensional model with fixed flux gave them the following cylinder growth rate:

$$\frac{dh}{dt} = \gamma = \frac{\mu}{q} \frac{I}{\pi a^2} = \frac{\mu}{q} \frac{4DC_0}{\pi a} \quad \text{Eq. 15}$$

Where  $h$  is the height of the colony,  $\gamma$  (gamma) is the cylinder growth rate,  $\mu$  is the cell growth rate,  $q$  is uptake,  $I$  is flux,  $a$  is the colony radius,  $D$  is diffusion, and  $C_0$  is starting glucose concentration. The cylinder growth rate depends on  $\mu/q$  which is the yield  $Y$ .

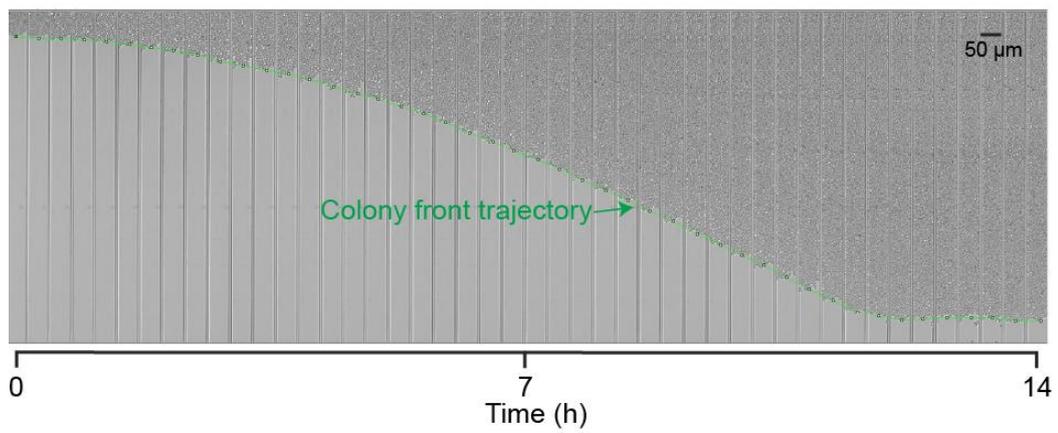
Since in this model they assumed fixed growth rate of the cells  $\mu$  and fixed uptake rate  $q$ , they did a numerical model and solved it for the case when  $\mu$  and  $q$  depend on the nutrient concentration. This model showed good correlation with cylindrical colony growth at glucose concentrations up to 1% (55 mM) but when it was based on previously published data for cell growth and uptake rate as a function of glucose concentration, the model did not capture the saturation of the colony growth rate at high glucose concentrations >2% (111 mM). However, keeping yield constant but modulating the uptake rate (the lower the uptake, the more layers of cells grow) brought them closer to capturing the behaviour in high glucose concentrations.

I outlined many difficulties and huge complexities that are involved in growth of cell populations but I did not address the unique identities of single cells that make these populations. The last thing I want to mention is the heritable traits, or better to say, genotype to phenotype relationships. There are three sets of factors that give rise to different traits in an individual: 1) genetic factors that lead to heritable differences in an environment, 2) environmental factors that modulate the effects of genotypes, and 3) the inherent variability of the trait even when genetic and environmental factors are identical [150]. To understand growth and behaviour of cells we need to understand how all these factors contribute to quantitative trait variation. With this we will be better at predicting phenotypes from genotypes and quantifying their inherent uncertainties. I particularly like the work from Ziv and his colleagues [150] because they were investigating the same model system I am using, yeast *S. cerevisiae*, and the glucose transporter gene HXT7 which plays a big role in most of my research endeavour. They looked at two different growth phases, lag

phase (in growth rate limiting glucose concentrations) and exponential phase (in growth rate non-limiting glucose concentrations), and tried to combine and quantify the parameters which characterize them: genotypes (phenotypic variation), environmental conditions (phenotypic plasticity) and phenotypic variability among isogenic cells in a given environment. As Gaël Yvert suggested, we need to treat every cell as unique and now with new technologies we can measure how genotypes shape the probability laws of single cell traits. Such an approach would require from us to think about phenotypes as probabilities, similar to a concept that statistical physicists have been applying to particles for a century [151]. Artémis Llamosi studied single-cell identity across many single-cell parameters and concluded that average behaviour of a population of cells doesn't correspond to average behaviour of a single-cell and that neglecting cell-to-cell differences biases our quantitative representation and understanding of the functioning of cellular systems [152]. Ziv and his colleagues discovered that some quantitative trait loci (QTL) are common between traits and environments and some are unique, which shows gene by environment interactions. For glucose transporter gene HXT7 specifically, they discovered that sequence variation contributes to variation in growth rate and lag duration. This sequence variation stems both from allele replacements of the entire locus and from single amino acid changes. Yeast tend to amplify HXT7 in glucose-limited environments and this is frequently selected in evolution experiments even though HXT7 amplifications result in antagonistic pleiotropy (an effect when one gene controls more than one phenotypic trait where at least one trait is beneficial for the organism and at least one trait is detrimental) in laboratory strains and not in naturally occurring variants. Their study is a good example of the complexity of the genotype to phenotype interactions within and between environments [150].



# Chapter I: Growing extended yeast colonies





## **Growth in microfluidic devices**

Studies of yeast colonies and their growth are mainly done on agar gels, as I presented before. The main drawback that arises from such studies is that it is virtually impossible to get a good resolution all the way to the single cell level since cells quickly produce thick layers. To circumvent that issue some groups have used methods like fixation and freezing so that they were able to dissect the colony in the middle and look at the inner colony structure with wide field or confocal microscopy [38]. While this can give many insights into internal organization of a colony, colony growth and cells cannot be tracked dynamically in time so this information is readily lost. However, by using microfluidics we can both observe cells dynamically in time for many days and get a good resolution of a colony all the way down to the single cells. In addition to this, environments can be imposed and seamlessly dynamically changed at experimenters wishes, as well as its shapes and dimensions. Not to mention the high throughput capabilities that lead to an easy quantification of phenomena.

Microfluidics is a powerful technique based on the production of micro-scale devices which can contain many chambers and channels for studies in biology, commonly known as a lab-on-a-chip. Since the invention of soft lithography [153], the potential of microfluidics has been recognized by microbiologists and other researchers in life sciences [154]. Collaboration between engineers, physicists, and biologists has pushed this field so far that today we can build devices of high complexity, all the way up to the so called organs-on-a-chip microfluidic devices that mimic mechanics and physiological responses of entire organs like hearts, kidneys, arteries, lungs, and skin [155], [156]. Today, microfluidics is used in many biology laboratories. Microfluidic chips are usually made with an elastomeric polymer polydimethylsiloxane (PDMS) is the most common method due to its properties like optical transparency, biocompatibility, chemically inertness, gas-permeability, flexibility and low cost [157].

In microbiology, the bulk of research is done with the consideration to provide all cells with a homogeneous environment. Even in microfluidics, flows of nutrients are imposed in such a way that all cells experience a homogeneous environment or they are kept apart from each other so that their properties do not produce undesired effects [158]–[161]. There are specialized devices that were built for gradient creation, mostly focusing on chemotaxis or pheromone gradients [162]–[165], but in none of them, as far as we know, the gradients were created by

cells themselves (Figure 30). As a recent review by Lin and Levchenko states: “The current, idealized spatial inputs used in many microfluidic devices (steady state gradients between a source and sink) could potentially be too artificial, and may elicit signalling and subsequent cellular behaviour which may substantially deviate from that *in vivo*” [166]. The majority of microbiology research in microfluidics that focuses on spatio-temporal variability is done in bacterial biofilms and researchers have observed nutrient gradients formed by the cells in thick biofilms [167]–[169]. In yeast, however, most of the research in microfluidics is performed on single cells or populations of cells in homogeneous environments [152], [170]–[174], and to the best of our knowledge no one has so far looked at growth of a monolayer of yeast colonies in gradients of nutrients imposed by the cells themselves. This is what researchers usually try to avoid and they try to build chip design features that allow homogenisation of the environment. **Since cells in the environment rarely live in such perfect conditions, exactly the opposite is in the focus of our interest.**

We want to build a microfluidic device in which we can load enough single yeast cells that they can, when grown together, “self-inflict” gradients of nutrients upon themselves and interact through “long-range metabolic interactions” within a colony, in which heterogeneous phenotypes (caused by the environment and the environment caused by them) of each isogenic single cell influence each other and create emergent properties of a colony through self-organization that would not otherwise be possible when living alone as a single cell. We think that a device like this would bring us closer to interactions and phenomena which can be found in nature (Figure 29).

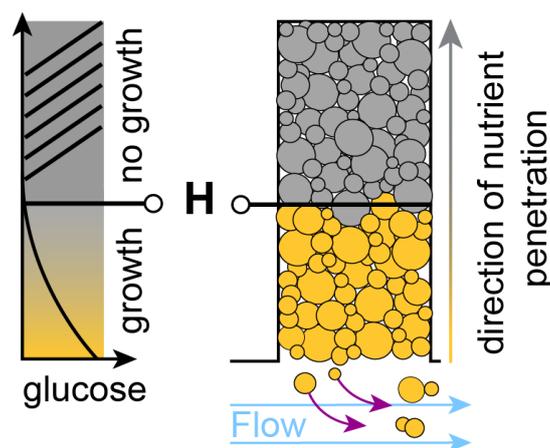


Figure 29 A sketch of a proposed model of a microfluidic device which could fit a monolayer of yeast cells at high enough cell numbers that we can observe a gradient of nutrients forming within a colony and differentiation into subpopulation that grow up to the point H, which marks the position of depletion of a nutrient essential for growth.

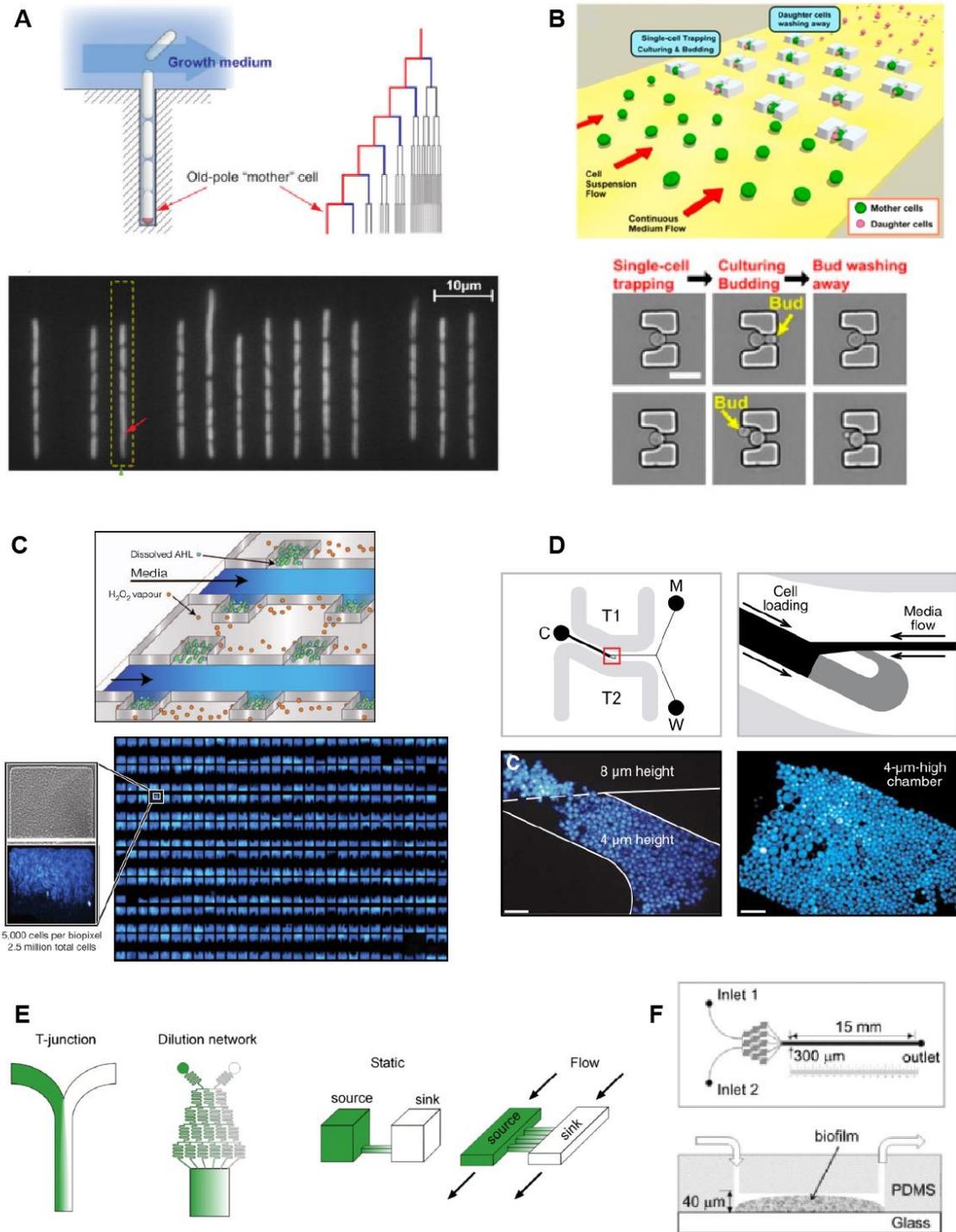


Figure 30 Microfluidic chambers for single cells or populations of yeast and bacteria. (A) The “Mother Machine” for aging, lineage, and single cell studies of *E. coli* [48]. (B) A device for aging and single cell studies in yeast [172]. (C) Synchronized *E. coli* “biopixels”, an array of chambers that can hold a population of bacteria in mostly homogeneous environment [175]. (D) The “Tesla chemostat” (inspired by a Tesla diode loop) used for studies of yeast populations on a single-cell level in homogeneous environment [176]. (E) Different devices for artificial gradient formation by parallel laminar flows or source to sink diffusion [166]. (F) Microfluidic device for growth of multilayered bacterial biofilms [177]. All figures were adapted from their referenced source.

## **Extended yeast monolayer can be grown in a microfluidic device**

We designed a multi-layered microfluidic device that we call the “Yeast Machine” (Figure 31). We gave it such a name in reference to the microfluidic device called the “Mother Machine” which has become a widely used tool in the *E. coli* community [48], [178], [179]. The “Mother Machine” was originally designed to follow steady state growth and division of a large number of *E. coli* cells at a defined reproductive age in a dead-end cell chamber that fit a single strip of bacteria perpendicularly connected to a flow channel. Our “Yeast Machine” is built on similar principles except that we wanted to grow a colony which consists of thousands of cells. It can fit several hundred micrometers long monolayer of yeast *S. cerevisiae* cells, long enough to observe variations of nutrient concentrations along its length. A large nutrient channel (1000  $\mu\text{m}$  wide, 25  $\mu\text{m}$  high) is used to flow nutrients which can diffuse into a perpendicular array of thin, long dead-end cell chambers (50  $\mu\text{m}$  wide and 800  $\mu\text{m}$  long) in which yeast cells grow. Cell chambers are typically 4.5  $\mu\text{m}$  high, comparable to the yeast cell size, so that cells are vertically constrained, facilitating single-cell imaging and fluorescence microscopy and giving a high content information on the spatio-temporal properties of a growing community of yeast cells. We first inject cells in the microfluidic system through the main channel and load them into the dead-end chambers by centrifugation using a homemade 3D printed device and a spin coater. After that we wash the main channel with yeast synthetic complete growth medium in order to remove the remaining cells. In contrast, cells in the dead-end chamber array are trapped. They start to grow from the dead-end of the cell chamber, progressing collectively towards the nutrient source due to geometric constraint and eventually fill out the chamber and form an extended two-dimensional colony composed of up to 2500 cells (Figure 32). Individual cells can be observed at high magnification (100x or 60x objective) and the entire assembly can be seen with a low magnification objective (10x objective). We then record a time lapse of the expansion of the colony and the spatial variation of fluorescence expression of key genes over several days.

The protocol for the “Mother Machine” fabrication, as well as a review of other chip designs that we tried can be found in the appendix under titles “Protocol for the microfluidic device fabrication” and “The steep path to the best chip design”.

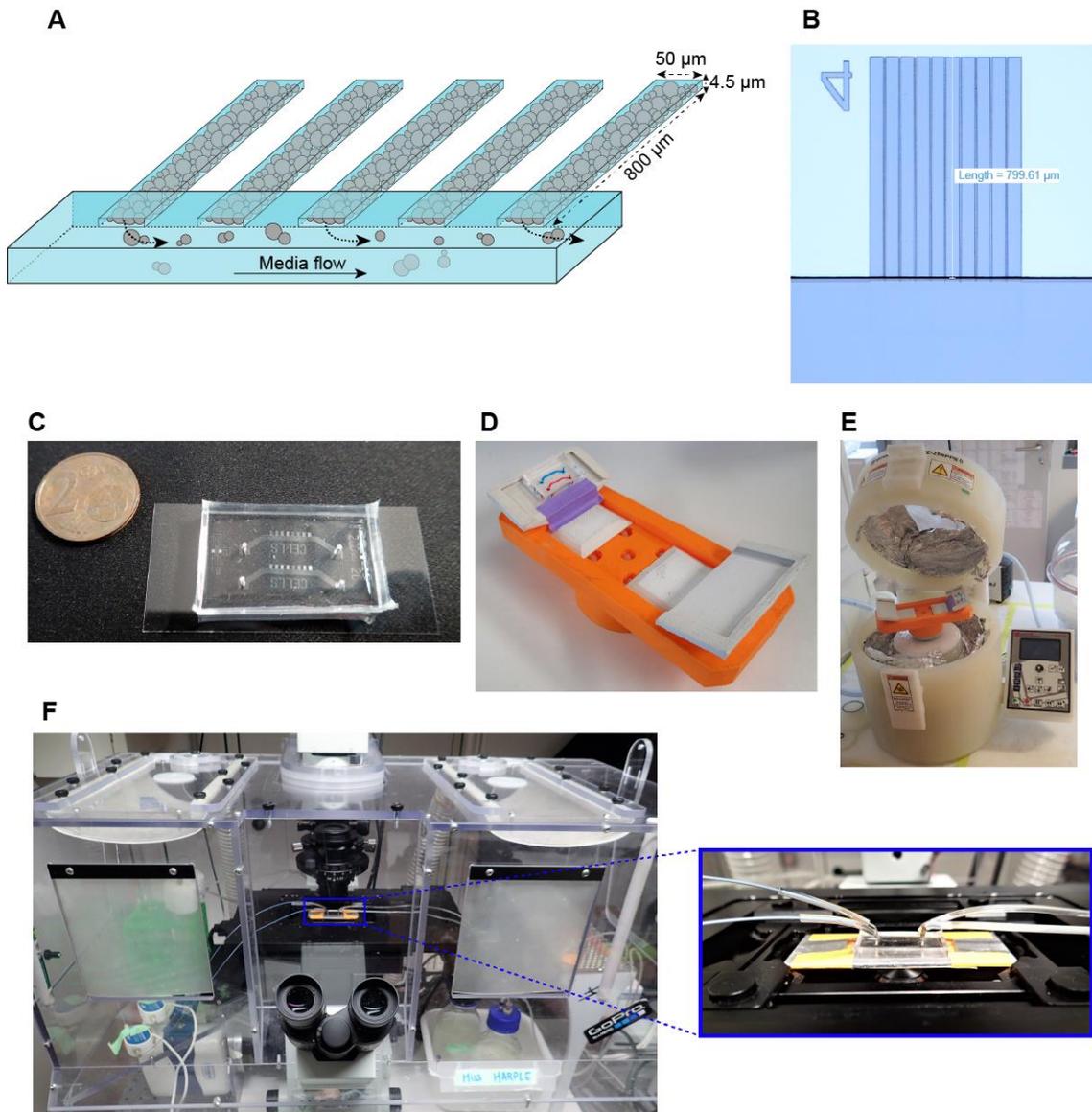


Figure 31 The “Yeast Machine” microfluidic device. (A) Sketch of the “Yeast Machine”. Big nutrient channel ( $1000\ \mu\text{m}$  wide,  $25\ \mu\text{m}$  high) is connected to a pressure based pump that pushes the nutrients through the channel and washes away the cells to the waste flasks. Cell chambers are connected perpendicularly to the nutrient channel. They are  $800\ \mu\text{m}$  long,  $50\ \mu\text{m}$  wide, and  $4.5\ \mu\text{m}$  high. A single cell chamber fits a monolayer of up to 2500 yeast cells. (B) A close up on an array of cell chambers connected to a nutrient channel. (C) Our microfluidic chip bonded to a cover slip. Each chip has two independent “Yeast Machines”. (D) 3D printed chip holder for centrifugation with the “Yeast Machine” mounted on it (channels are coloured with different dyes for better visualization). (E) Spin coater that we use for centrifugation of cells into the cell chamber. (F) Microfluidic chip mounted on a microscope.

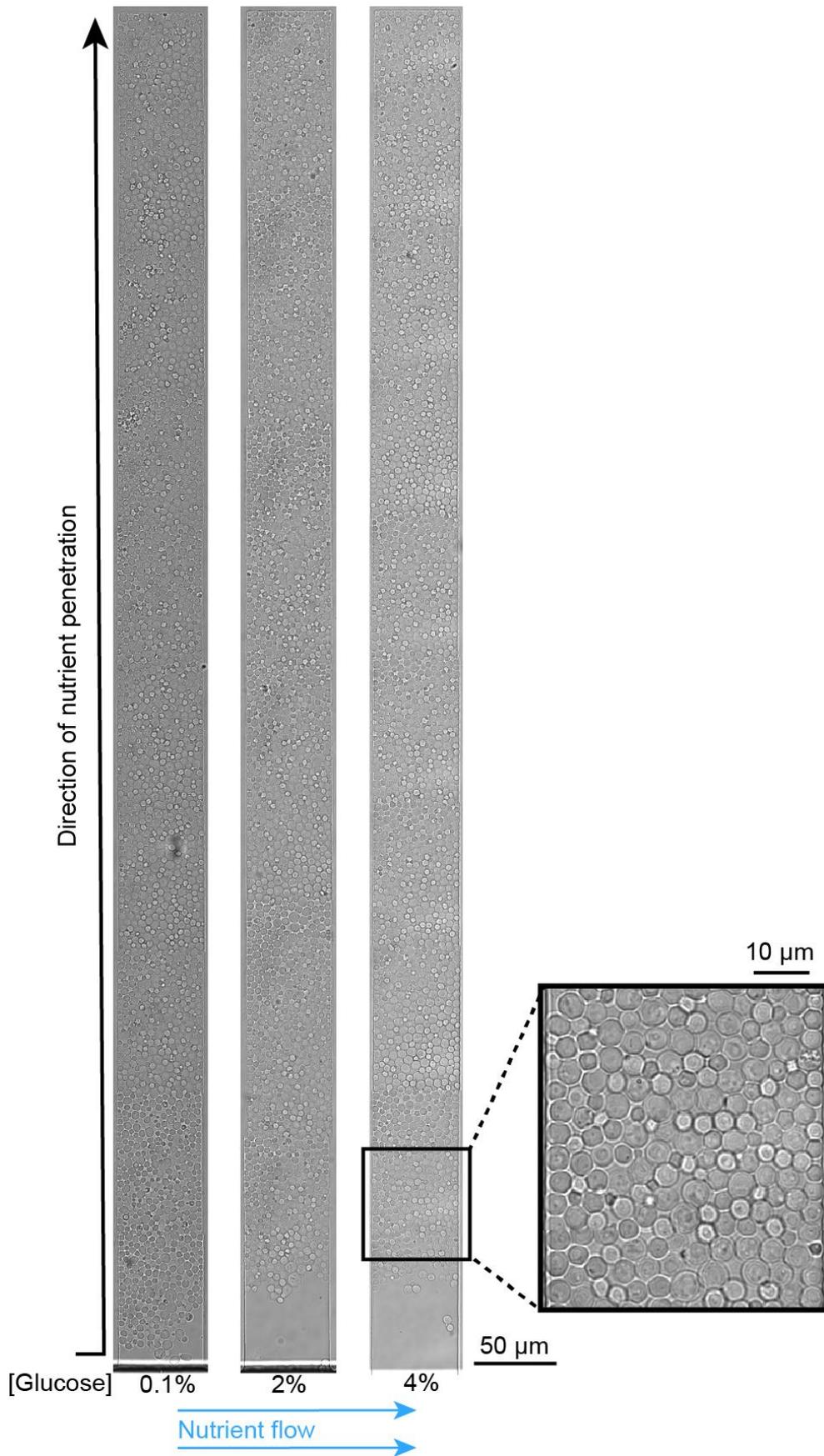


Figure 32 Cell chambers of the “Yeast Machine” at high magnification in different starting glucose concentrations.

### Method for cell front tracking and calculating colony front velocity

We performed all image analysis in an open source software ImageJ [180]. Image analysis of the cell front tracking was done in collaboration with Xiaohu Song from INSERM U1001. Cell tracking in our work is an essential tool to detect the motion of cells because it is necessary that we quantify growth of the whole colony. To obtain the front velocity of the colony which is defined by the difference of the frontier position divided by the time difference, we apply a threshold defined by Otsu to detect the bottom frontier through time after flattening the background by a FFT band-pass filter [181]. In practice, we select a colony that we want to measure and apply this method. Each timeframe is then pasted into one image resulting in a kymograph where we can see how colony grew in time (Figure 33 A). We can manually adjust cell front position to correct for all the mistakes in image analysis. From this we obtain a colony front trajectory and use it to calculate the colony front velocity (Figure 33 B). Colony front velocity is a derivative of the colony front position in time and it is calculated for each single timestep producing many velocity points that we then bin and average out (Figure 33 C).

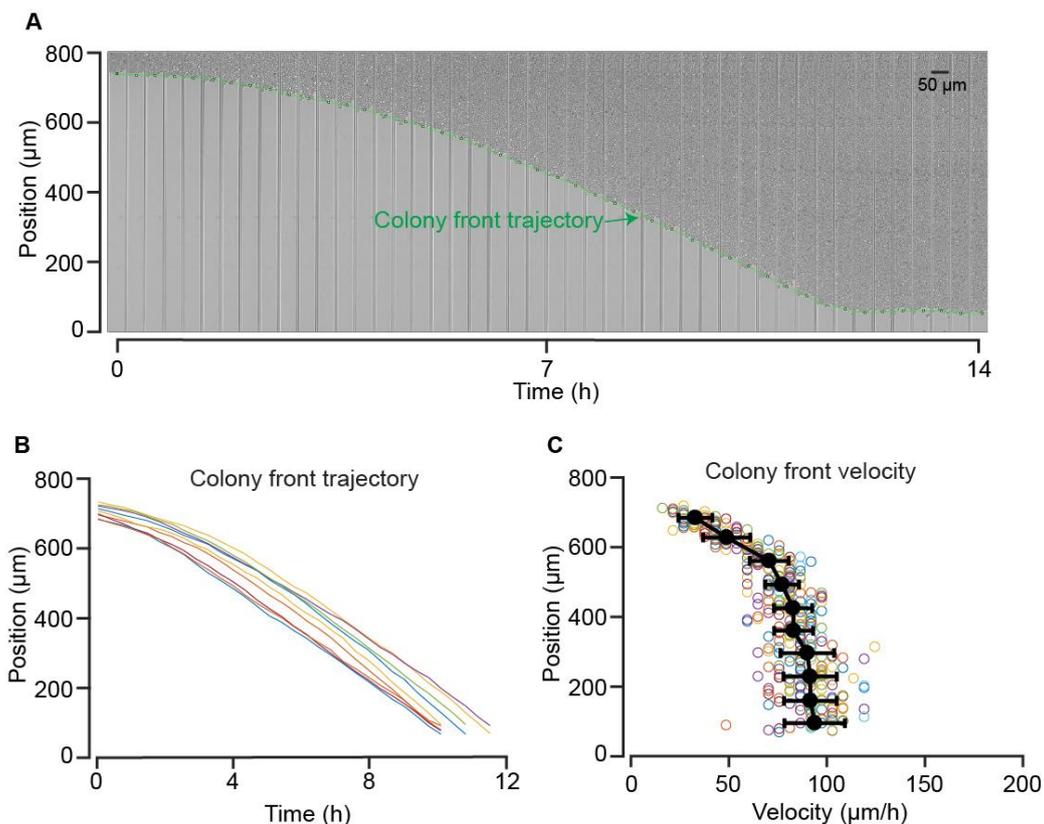


Figure 33 Colony front trajectories and velocities. (A) A kymograph of colony expansion in time. Position 0  $\mu\text{m}$  is the position of the glucose source. (B) Extracted and combined colony front trajectories of many different colonies. (C) Colony front velocity calculated as derivative of the trajectories, binned, and averaged out.

## **In long chambers, cells create and experience a spatially structured microenvironment**

When we load the cell into the “Yeast Machine” usually only around one fifth or less of the cell chamber is full. As cells grow and divide they push each other in the direction of the nutrient channel where they are washed away by the flow (Figure 33). The expansion of the monolayer of cells can be measured by microscopy at low magnification and the velocity of the front can be extracted in function of time. By measuring the velocity of the cell front we can quantify the growth of the colony as a whole. In standard glucose rich conditions (2% glucose; 111 mM), the front velocity increases with time, as more and more cells participate in the colony expansion, and eventually reaches a plateau around 93  $\mu\text{m}/\text{h}$ . After some time, each dead-end chamber is filled with cells. During the expansion, the front velocity depends on how far glucose (and other nutrients) penetrates inside the colony (affecting the number of cells that grow and divide, and affecting the rate of growth and division of each cell). Let us take the example in 2% glucose and consider an ideal case when all the cells in a colony have the same growth rate, irrespective of possible glucose gradients forming. Yeast cells near the front are typically 4  $\mu\text{m}$  wide and if we assume that they typically divide in 90 min, this would mean that the velocity front can be attributed to the first 35 layers of cells, that is to the first 140  $\mu\text{m}$  of the colony. Yet, this too simple reasoning does not take into account the fact that cell growth rate decays when the glucose concentration decreases inside a colony. Also, the gradient shape depends on the specific uptake of glucose, which varies with the glucose concentration [90], [91], [97], [182], [183]. The real glucose penetration distance is therefore likely to be larger and difficult to predict based on our limited understanding of the internal structure of yeast assemblies. One could estimate the penetration distance assuming that glucose freely diffuses from the nutrient channel into the dead-end cell chamber with a diffusion coefficient  $D \sim 600 \mu\text{m}^2/\text{s}$  [184], [185] and that glucose, which concentration is maintained at  $C_0$  in the nutrient channel, is absorbed at a constant rate  $q \sim 5 \text{ mol s}^{-1} \text{ m}^{-3}$  [182]. Diffusion law tells that the glucose concentration is expected to decrease significantly after a typical distance  $H$  that scales with  $\sqrt{DC_0/q}$  [149], that is in lower hundreds of micrometers. This competition between diffusion and uptake is central to the structuration of the colony since it affects both the number of cell layers that have access to glucose and the glucose concentration in the microenvironment of each layer, determining which cells actually participate in colony expansion and by how much. We also anticipate that the microenvironment landscape will trigger differential gene expression according to the location of cells, namely their distance

from the nutrient source. In all figures position 0  $\mu\text{m}$  is the position of the nutrient source.

Combining results across almost a 1000-fold range of glucose concentrations (from 0.01% to 8% glucose; 0.55 mM to 444 mM) we can see how colonies grow in each case. If we assume that there is a certain distance  $H$  at which there will be no more glucose because cells depleted within a colony, we should see a levelling off of the front velocities once the number of cells that grow and their growth rates become fixed in the colony. We can see this in higher glucose concentrations while in the lower starting glucose concentrations there seems to be a very slight linear increase in colony front velocity as cells are growing although we would expect that the front velocity fixes very quickly because the growth should be driven by a small number of cell layers, something that can be seen in the case of 0.05% glucose (Figure 34).

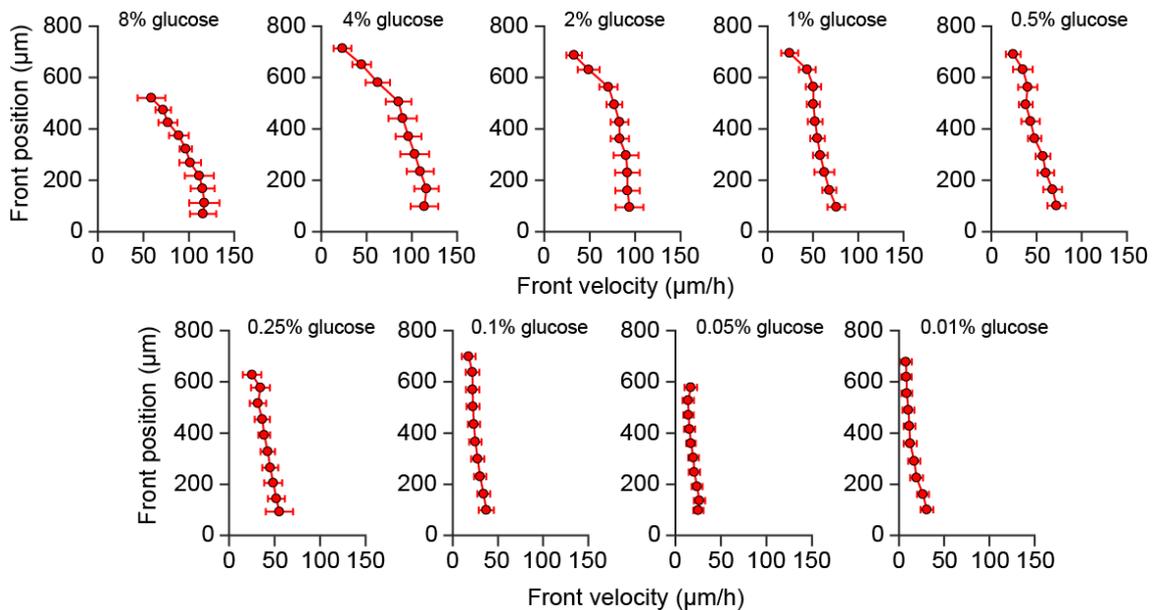


Figure 34 Colony front velocities. Each panel represents colony front velocity at each position measured while colonies were growing in the “Yeast Machine” across a range of glucose concentrations. Depending on the starting glucose concentrations, but also diffusion and glucose uptake rates which evolve as function of local glucose concentration, the number of cell layers that grow and their growth rates are set.

A better way to visualise and understand what is going on in the colony is to plot the colony front velocity as a function of glucose concentration (Figure 35 B). The measurement of the front velocity in this case is taken once the colony expanded the most in the 800  $\mu\text{m}$  long chamber. We can see that the colony front velocity increases as we increase starting glucose concentration from 25  $\mu\text{m/h}$  in low glucose concentrations up to 116  $\mu\text{m/h}$  in high glucose concentrations. However, at

4% glucose concentration the front velocity levels off. This is mostly due to the finite size of our colony, meaning that at 4% glucose most of the cells participate in growth. We know that the growth rate of *S. cerevisiae* quickly saturates in glucose [91], [97], [182], [186], [187] (Figure 35 A). For example, in Mark Siegal's lab they define a growth rate limiting media at 0.004% (0.22 mM) glucose and growth rate non-limiting media already at 0.08% (4.44 mM) glucose claiming that the latter concentration of glucose supports maximal growth rates, although it is more than an order of magnitude lower than that contained in standard lab media (2%; 111 mM) [150]. If there is glucose everywhere at 4% starting glucose concentration and since growth rate quickly saturates, adding more glucose should not affect the front velocity. Once glucose is depleted at a certain distance in the colony, front velocity should be slower, like we see in  $\leq 2\%$  glucose examples.

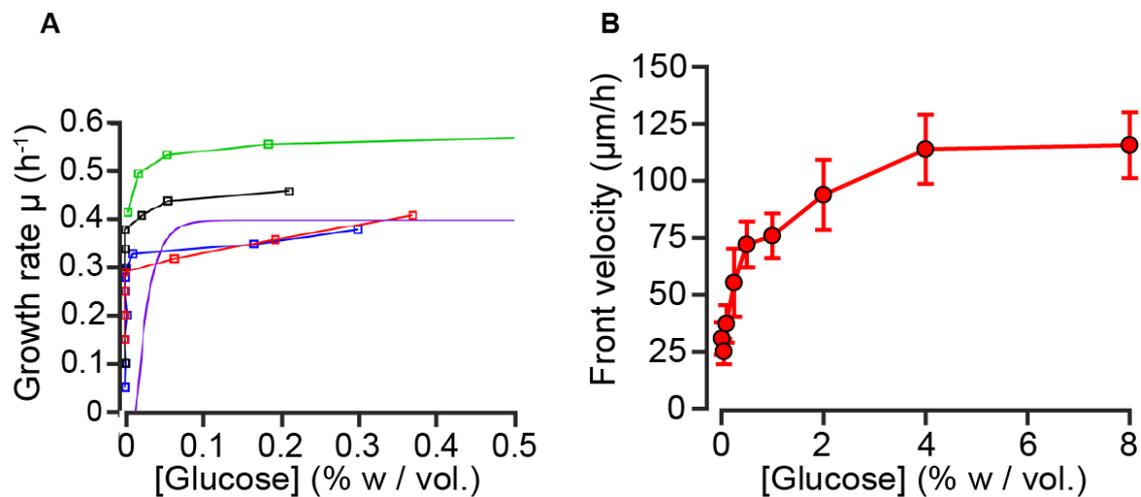


Figure 35 Single cell and colony growth. (A) *S. cerevisiae* growth rates in liquid cultures as measured by Youk (green) [182], Postma (black) [186], Reifenberger (purple) [91], Diderich (blue) [97], and Rieger (red) [187]. Figure adapted from [13]. (B) Colony front velocity as a function of glucose concentration.

A quick way to visualise motion of the cells is to use the projection of pixel values in time (Z projection function in ImageJ). We compute the standard deviation of pixel intensity pixel by pixel. If the pixel values changed in time (meaning that there was movement of cells) the values of the standard deviation will increase. From this data we can visualize and measure where the cell movement occurred. Unfortunately, this method is not precise or sensitive enough to measure the growth rates along the whole colony (because even a small movement will result in high standard deviation value and cause false positives) but it is good enough to distinguish between growing and growth arrested parts of the colony. Dark pixels

represent no movement, while white pixel represent movement (Figure 36 C, D). In the example of 1% glucose we can see that the cell motion starts to rapidly decrease around position of 300  $\mu\text{m}$  away from the glucose source due to the lack of nutrients. Schematically we can imagine that glucose concentration decreases as a function of the position from the glucose source and at one point reaches zero, causing the cell growth arrest and effectively forming differentiation in the colony between growing and non-growing cells (Figure 36 A, B).

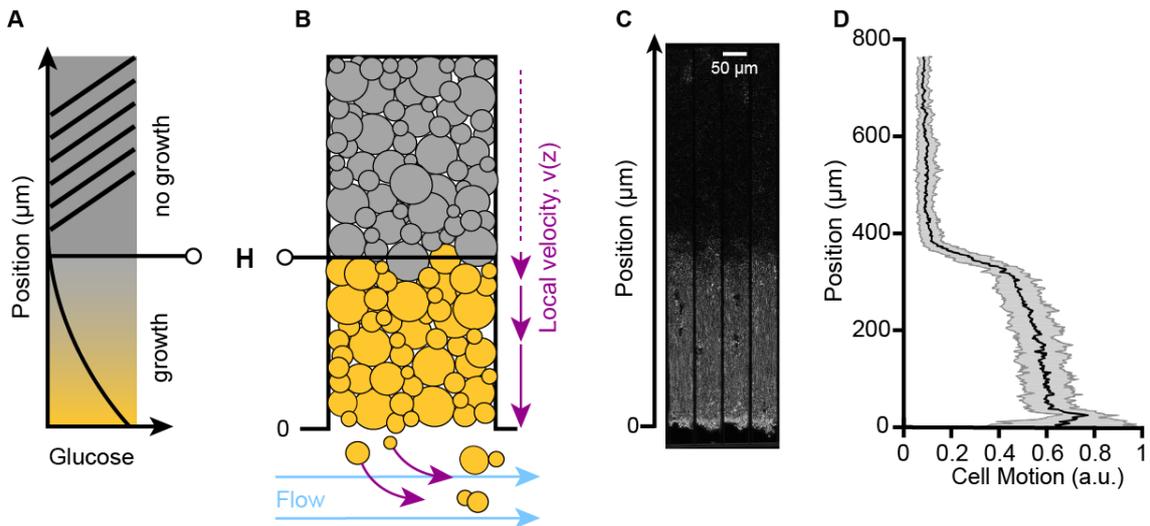


Figure 36 Differentiation between growing and non-growing cells in a colony. (A) Glucose gradient inside a colony is formed due to the interplay of diffusion and glucose uptake by the cells. At one point glucose is depleted and cells cannot grow. (B) This causes differentiation inside a colony between population that grows and population that doesn't grow. (C, D) Cell motion caused by growth can be visualized by projecting standard deviation of pixel intensity values in time. Black pixels represent no motion and white pixels represent motion.

While it is useful to visualise motion of the cells in a colony or look at the evolution of the colony front velocity in time as it grows from a few dozens of cells (although this is limiting in time due to length of the cell chamber, so one might imagine pushing the boundaries and making longer channels exclusively to this purpose), our interest goes beyond this. I will now focus at a time after a colony is established, when it fills out the whole cell chamber and grows inside. Already at the scale of a few hundred micrometres it differentiates into two subpopulations that grow and do not grow at glucose concentrations commonly used in the lab, making it relevant for the study of colony growth.

### ***Field velocity within a colony***

We have seen how the colony front behaves in time and in different glucose concentrations, how it grows as a whole, but what happens with the cells inside? Can we try to figure out growth in each layer of the colony and see how the growth rate of each cells contributes to the growth of the whole colony?

In our system cells are pushing each other toward the nutrient source. This means that all cells that are growing contribute to the velocity of cells that are closer to the entrance of the cell chamber. Cell close to the area where glucose is depleted move slower, while cells close to the nutrient source move faster because they are pushed by all the cells between them and the no glucose border. We call this the local velocity  $v(z)$ , that depends on the position  $z$  in a colony (Figure 36 B). Said differently, local velocity is the integral of all cellular growth rates between the no glucose (or even better said – no growth) border  $H$  and the local position  $z$  in question. We can formalise this with the following equation:

$$v(z) = \int_0^z \mu(z') dz' \quad \text{Eq. 16}$$

Where  $v(z)$  is the local velocity at position  $z$  and  $\mu(z')$  is the growth rate at position  $z'$ . If we apply this equation to the front velocity we can see that the front velocity should enter in a linear expansion regime once cells stop dividing at the height  $H$  (since  $\mu = 0$  for  $z > H$ ). When cells are not limited by glucose and  $\mu(z) = \mu_{\max}$ , the colony should grow exponentially. However, if “added” cells participate less and less in the growth of a colony as a whole, the colony should slowly turn to linear growth.

We used two different methods to quantify local velocities. First one is a function in ImageJ [180] that is called “Orthogonal views”. It produces a kymograph along one pixel wide line and plots the pixel values along the line as a function of time (Figure 37 A). Since cells are not of uniform colour and one can distinguish membranes from the cell interior (at 10x magnification), we can visualise lines of different grey shades moving in time. It is a descent method for visualizing and measuring slow cells or cells that do not move but it is not so good when cells start to move quick because the lines get blurred out. This is why, for our final data, we used an open source image analysis tool called “TrackMate” [188]. This software can detect “particles” and then follow them in time. There is always a certain number of cells that are fluorescent in red channel. Even more so when they are damaged or dead. So, we used red fluorescence channel to image cells and used them as particles that we can track and extract velocities from. As

a cell can be miss-detected in a very noisy environment like ours at low magnification, the prediction of the future position based on the previous motions needs to be considered too. “TrackMate” achieves this purpose well. The essential part of this software is to use a Kalman Filter [189] to predict the future motion and use the Linear Assignment Problem (LAP) [190] to minimize the matrix cost to achieve the global optimization of tracking. Such modularized design of this plugin allows us to integrate the detection method based on Point Spread Function [191] which enables us to obtain a better detection of cells (Figure 37 B, C).

In practice, we load a time-lapse microscopy movie and run “TrackMate” by choosing parameters suitable for detection of particles of our size and motion. Since the motion of the cells through the cell chamber is not always following a straight line (mostly due to a cell, or groups of cells being stuck at a certain position in the chamber) we manually remove such trajectories. There are two different modes: automatic tracking and semi-automatic tracking. In our hands, both modes give very similar results after removal of bad trajectories. Semi-automatic tracking is more labour intensive because one needs to choose each cell one by one for tracking and automatic mode tracks all the cells at once but asks for more work on the removal of bad trajectories.

After processing we obtain cell trajectories and use them to calculate the local velocity within a colony (Figure 37 D). Local velocity is a derivative of a position of a cell in time and it is calculated for each single timestep producing many velocity points that we then bin and average out (Figure 37 E). There is a big variability of velocities that stems both from the method, from non-perfect motion of cells, and surly from experiments and cell variability too. We tried to apply smoothing to the trajectories, which resulted in almost identical mean velocities but somewhat smaller standard deviations. Nevertheless, all the data that I will present here will be data with no smoothing but rather all the trajectories and the velocities come from the obtained raw measurements.

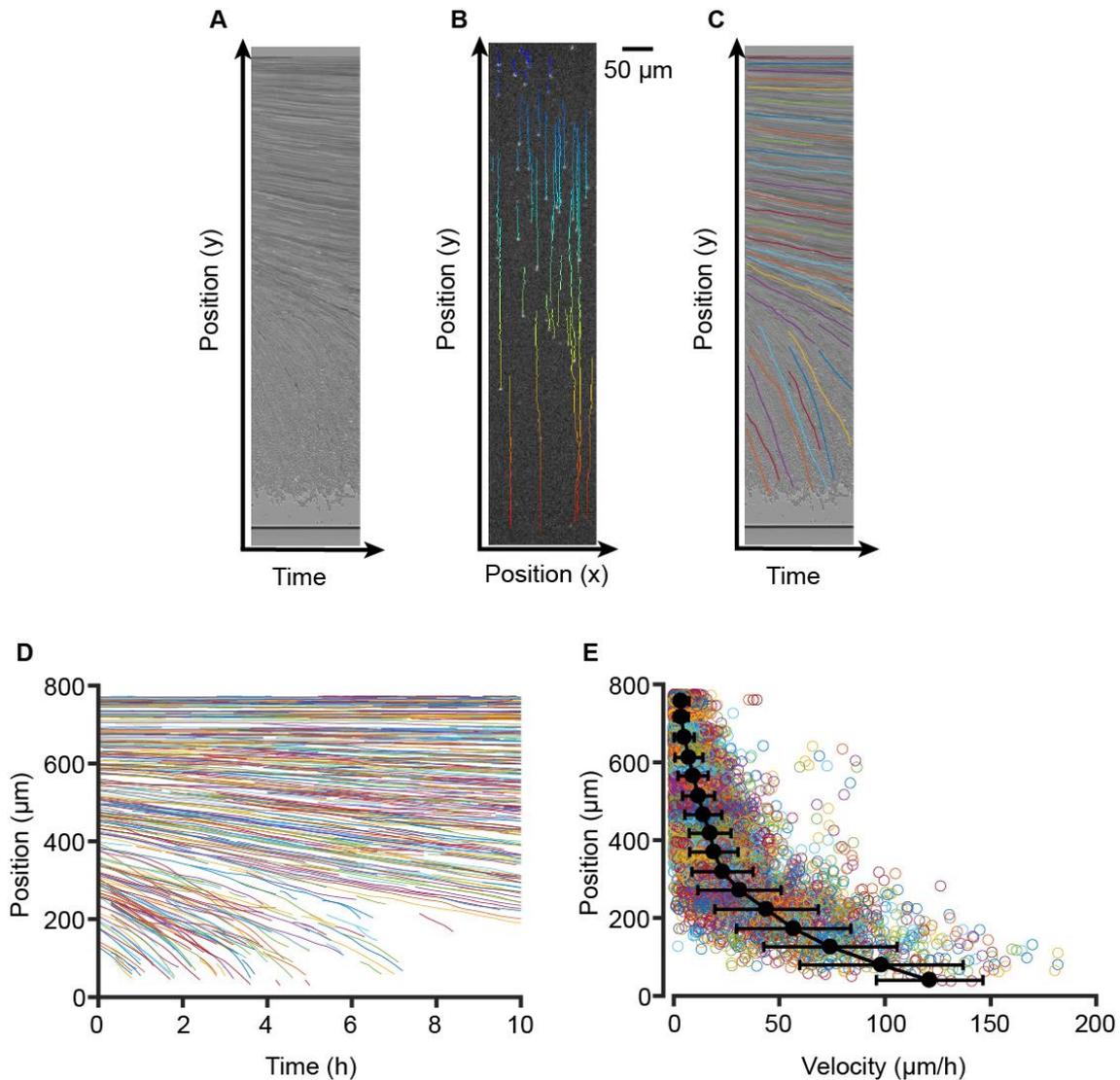


Figure 37 Single cell trajectories and local velocities. (A) A kymograph of section of a colony only a pixel wide obtained with “Orthogonal view” function in ImageJ. Characteristic lines emerge that we can quantify to get local velocities within a colony. (B) A more accurate method of measuring local velocities. Cells that fluoresce in red fluorescence channel can be tracked with help of the “TrackMate” software. (C) Kymograph with superimposed cell trajectories. (D) Cell trajectories of hundreds of cells in experiments with 4% starting glucose concentrations. (E) Local velocities at each position in a fully-grown colony. Position 0 μm is the position of the glucose source.

## Local expansion rate decreases with the distance from the nutrient source

The microenvironment landscape inside a colony is maintained at later time, when cells fill the whole dead-end chamber. The first evidence of this microenvironment landscape comes from the analysis of the local division rates of

cells. We first looked at the growth rate of cells in an array of 800  $\mu\text{m}$  long chambers with starting glucose concentration  $C_0 = 111 \text{ mM}$  (standard 2% glucose conditions). We also used a high concentration of amino acids (five times more than in a regular well-defined media) to make sure that amino acids were not the limiting nutrient. We observed that cells near the chamber's dead-ends ( $z \sim 800 \mu\text{m}$ ) did not grow nor divide anymore except in 8% glucose and the most of cells grew and divided in 4% glucose too (Figure 38). Conversely, cells closer to the source ( $z \sim 0 \mu\text{m}$ ) showed active growth and fast and regular cell division. This observation demonstrates that there is a natural organization emerging from the establishment of a glucose gradient. Interestingly, cells that grow push each other towards the entry into the nutrient channel due to geometric constraints. The motion of a cell is thus the result of the cumulative growth of cells that are placed above it relative to the nutrient source. As a result, a cell at a given position moves faster toward the nutrient source than the cells above it. We did measure the velocity field in standard conditions and extracted 100-500 single cell trajectories per condition resulting in thousands of velocity data points. As expected the velocity increases when cells get closer to the entry into the nutrient channel. Increasing glucose concentrations (from 0.05% to 8%) led to larger local velocities and larger front velocities, in agreement with the fact that glucose can penetrate further in the colony. If all cells were growing at the same rate, the local velocity should scale as the distance squared. This is not what was observed experimentally.

Let us first compare local velocities in the highest (8%) and the lowest (0.05%) starting glucose conditions (Figure 38). In 8% glucose all cells in the cell chamber grow, while in 0.05% glucose only cells from the position of the glucose source at 0  $\mu\text{m}$  up to about 100  $\mu\text{m}$  grow. Cells close to the glucose source also move significantly faster in 8% glucose due to the growth of the whole colony where cells push each other in the direction of the glucose source. As the starting glucose concentration increases also the number of cells that are growing increases. Also with that the local velocities within a colony increase too. It is interesting to see that there is not much difference between 1%, 0.5%, and maybe even 0.25% glucose. This might indicate a much more complex interplay between diffusion, glucose uptake rates, growth rates, and starting glucose concentrations than we expected. We can also never exclude influence of other nutrients or inhibitory metabolites that might modulate local velocities within a colony. When we plot local velocities in the three-dimensional space of positions, local velocities, and glucose concentrations we get a better intuition for the evolution of this complex landscape (Figure 39).

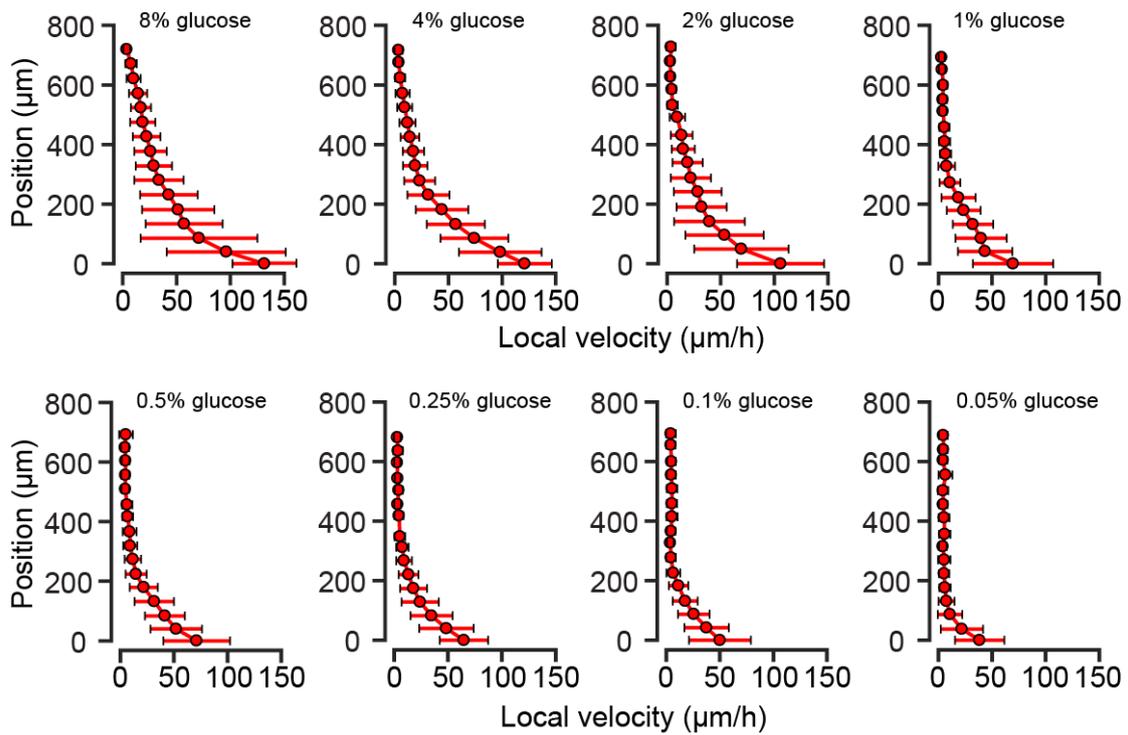


Figure 38 Local velocities within a colony. Each panel represents local velocity within a colony at each position measured while colonies were growing in the “Yeast Machine” across a range of glucose concentrations. Depending on the starting glucose concentrations, but also diffusion and glucose uptake rates which evolve as function of local glucose concentration, the number of cell layers that grow and their growth rates are set. Position 0  $\mu\text{m}$  is the position of the glucose source.

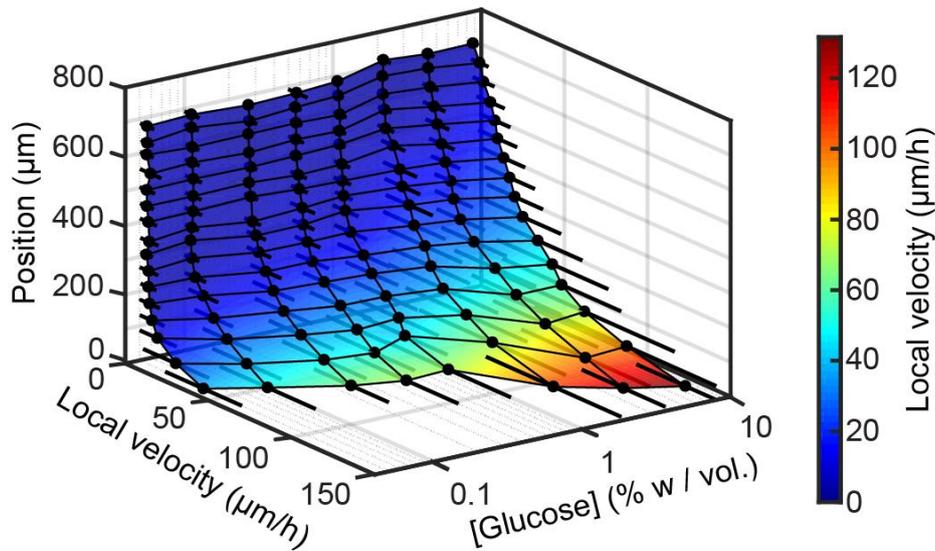


Figure 39 Local velocity landscape as a function of position and starting glucose concentrations. The higher the starting glucose concentration and the closer cells are to the glucose source, the higher local velocities will be.

Since local velocity at a certain position  $z$  in a colony is a product of integration of growth rates of all the cells between this position  $z$  and cells that contribute to the movement up to the point where there is no more growth, the local growth rate can be estimated from the spatial derivative of the velocity vector field that is obtained from single cell trajectories. We were thus able to infer the local growth rate as a function of the position of the cells in the channel and their distance from the nutrient source. In agreement with the existence of a glucose gradient we observed that the growth rate is decreasing with the distance to the glucose source (Figure 40). We first did a fitting of local velocities with an exponential function with two terms ( $f(x) = a \cdot \exp(b \cdot x) + c \cdot \exp(d \cdot x)$ ) and then from this fit we derived the local growth rates.

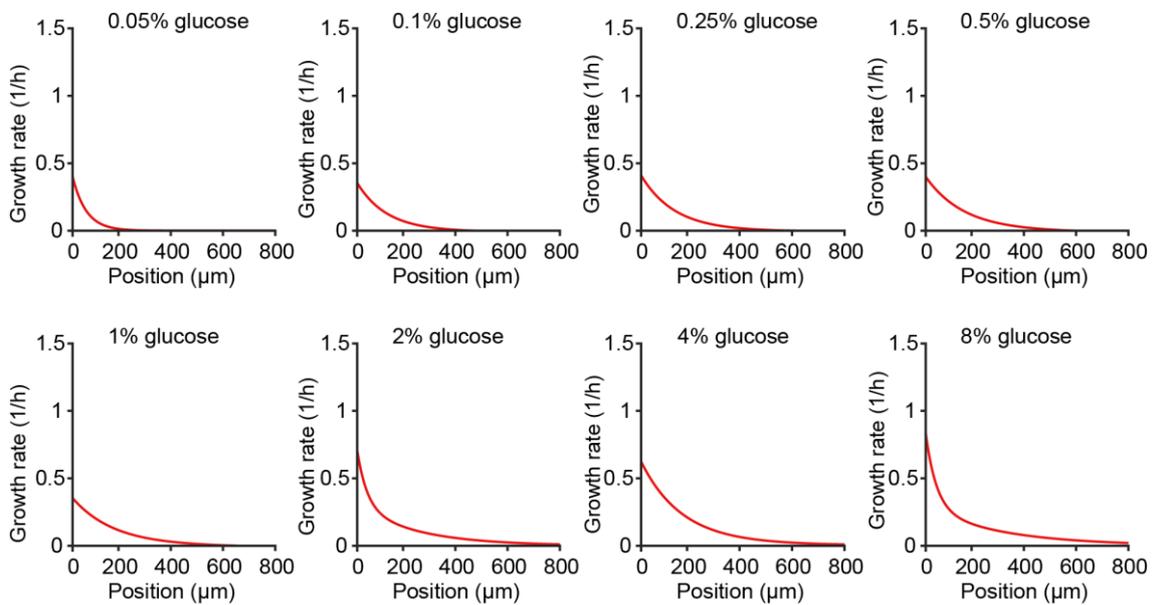


Figure 40 Growth rates within colony in different glucose concentration. Some regularities can be seen, the growth rates are diminishing deeper in the colony and there are differences in between different glucose concentrations, but not all of them.

Taken together, our results demonstrate that 800 μm long colony is long enough to observe the emergence of spatial variation of the cell local growth rate as a result of nutrient gradient formation (Figure 41). This gradient of glucose exists because of the cell metabolic activity which in turn impacts the cell growth rate and physiological state on a local level and the colony properties on a global level. Therein lies the origin of self-organization in multicellular assemblies that we are quantitatively investigating here.

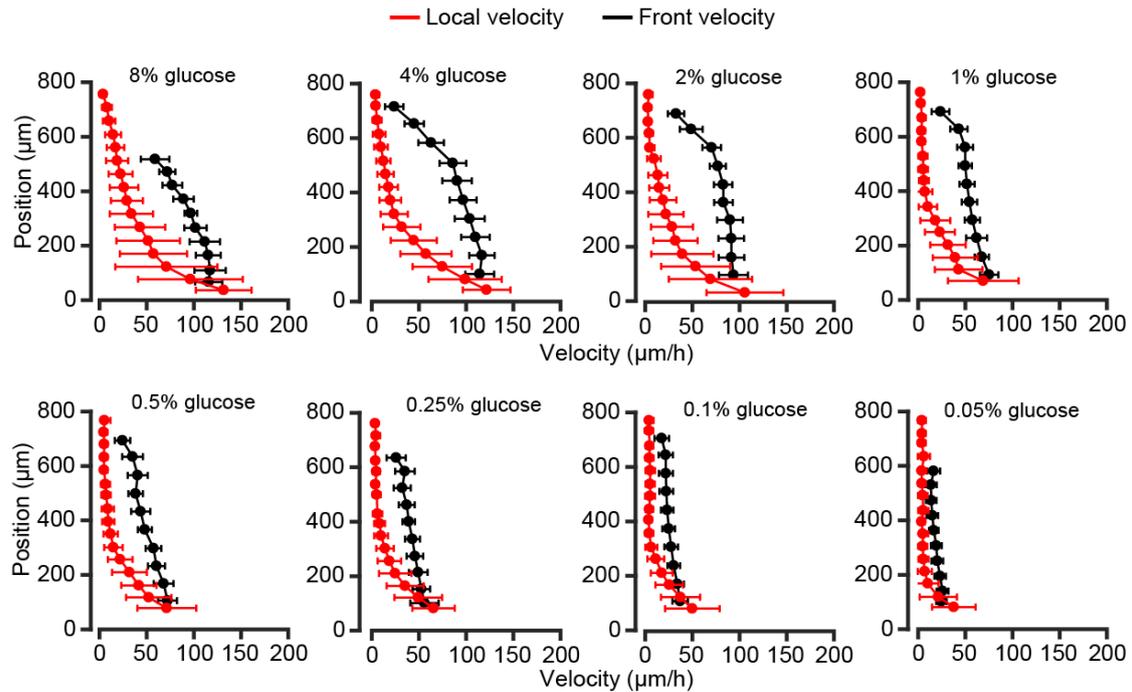
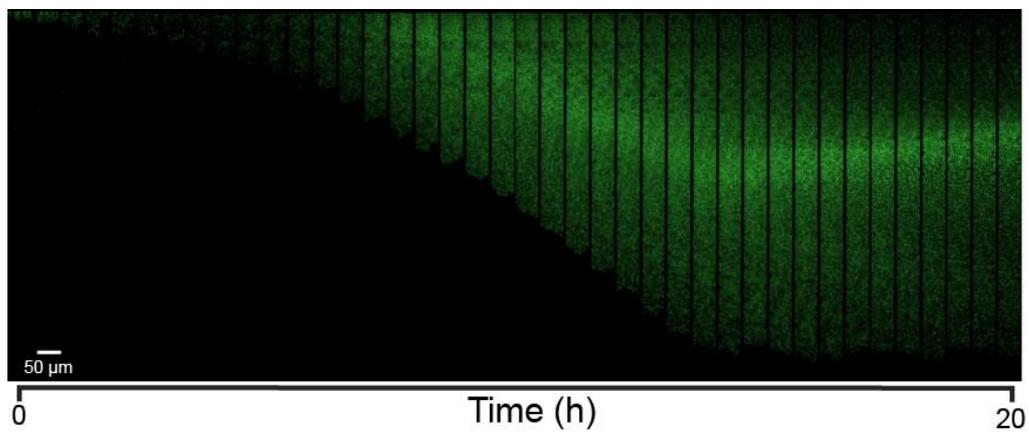


Figure 41 Colony front and local velocity comparison. Front and local velocities are hardly comparable except maybe up to a point when front velocity is supposed to become constant due to the constant number of layers of cells growing. From this point and up to the point when cells form a full colony, front velocities should be comparable with local velocities at the position closest to the glucose source. We can see that they are comparable for each glucose concentration. Possible variation could come from measurement inaccuracies, but also from the time of the measurement and adaptation of cells to new environments which doesn't happen in exactly the same way. For example, while colony is expanding in the cell chamber, cells at the front are in principle constantly in the same (starting) glucose concentration. On the other hand, once a whole colony is established and cells closest to the glucose source are constantly pushed, washed away, and the front is replenished by the cells from the back of the colony that need to adapt to increasing glucose concentrations.

Since we observed such a heterogeneous response from yeast when it comes to growth, we wondered next if we can see differentiation that could be linked to glucose concentrations, and if we can somehow extract the information on how much glucose there is in the environment. Moreover, we wanted to understand what is setting possible glucose concentrations gradients and how. This is why we decided to study glucose transporters in yeast, since they are responsible for taking up glucose from the environment. In the next chapter, I will focus on the gene expression landscape of glucose transporters.

## Chapter II: Landscape of gene expression in yeast colonies





## **There is a landscape of gene expression at the scale of the cell colony**

The discovery that we can use green fluorescent protein to tag other proteins and use it as a marker for gene expression inside cells was dawn of a new exciting era in cell biology [192], [193]. With this technique we can use live imaging of cells to track the level and localization of any protein we desire. This gives us an unprecedented insight into self-organization and differentiation of cells and cell populations. This is why we decided to map the gene expression of key genes that participate in glucose metabolism, with hope that we will be able to better understand what happens within a colony and how different features of the metabolism participate in structuring a colony.

We first decided to look at the level of expression of key glucose transporters, which expression level is known to be dependent on the concentration of glucose in the extracellular environment (HXT1-7). We used S288c based strains with glucose transporters endogenously tagged with GFP. Level of their expression can help us understand how, where, and how much glucose cells uptake from their environment.

### ***Flow cytometry experiments with glucose transporters***

We first measured the level of gene expression of HXT1-7 glucose transporters in batch as a function of glucose concentration. The protocol was as follows:

Flow cytometry experiments were performed on the Gallios Flow Cytometer (Beckman Coulter) equipped with 10 colours, 4 lasers (488nm Blue & 561nm Yellow [co-linear], 638nm Red, 405nm Violet). Excitation laser at 488 nm and emission filter FL1 (530/30) was used to detect the GFP fluorescence levels. Data analysis was done in Kaluza Flow Cytometry Analysis Software (Beckman Coulter). Approximately  $10^4$  cells were inoculated in 10 mL of Synthetic Complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids (BD Difco) + 0.79 g/L complete supplement mixture (CSM; MP Biomedicals)) containing log<sub>2</sub> dilutions of glucose (VWR) concentrations (from 8% to 0.0078125%, and 0% glucose) and

grown in a shaking incubator at 30°C for 16-18 hours reaching an OD600 ~0.02-0.2 depending on the starting glucose concentration. Cells were then diluted 10 times in 10 mL of fresh SC media containing the same starting glucose concentration and grown for 4-5 hours in a shaking incubator at 30°C. Cells were centrifuged at 4000 rpm for 10 minutes, resuspended in 300µL of PBS pH 7.4 buffer (Gibco) and their fluorescence was measured on the flow cytometer.

In order to quantify the gene expression as a function of glucose concentration it is important that cells are kept and measured in relatively constant glucose concentration. But cells consume glucose from the environment in a batch culture, so we designed the protocol very carefully to keep the glucose concentration as close to the starting glucose concentration as possible. We kept the glucose concentration in the media at the moment of the fluorescence measurement at around 90% of the starting glucose concentration (Figure 42). We collected the supernatant of each sample and measured glucose concentration with the Glucose (HK) Assay Kit (Sigma) to check how much glucose was left in the media just before the fluorescence measurement. The kit is based on NAD reduction to NADH during a reaction cascade catalyzed by hexokinase and glucose-6-phosphate dehydrogenase which leads to an increase in absorbance at 340 nm that is directly proportional to the glucose concentration.

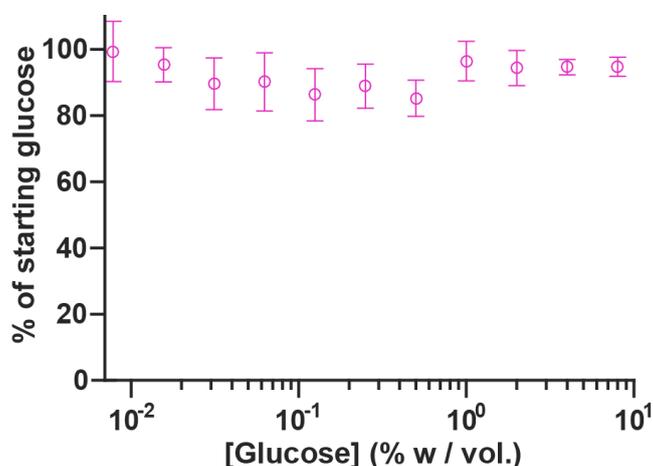


Figure 42 Percentage of the starting glucose concentrations at the moment of flow cytometry experiments. Cells consumed approximately 10% of starting glucose concentration.

As a negative control we used the background S288c BY4741 wild-type strain that has no fluorescent proteins. As a positive control we used galactose inducible strain that has GFP under the control of strongly inducible galactose promoter.

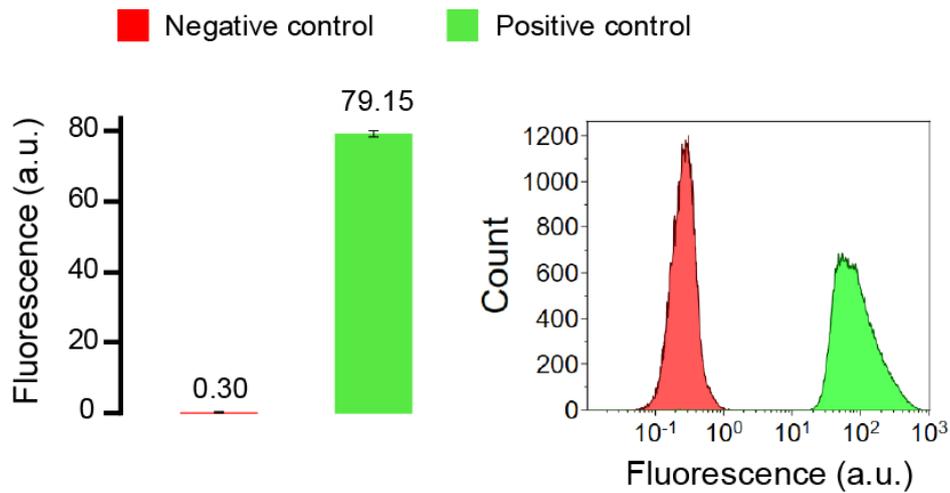


Figure 43 Positive and negative control for flow cytometry experiments. Left: average fluorescence. Right: fluorescence histograms.

We obtained different profiles showing that each of the seven transporters is expressed and repressed at different glucose concentrations (Figure 44). As expected from the literature [182], [194], HXT1 and HXT7 have a prominently opposite expression pattern (Figure 45). HXT1 is a low affinity glucose transporter expressed in high glucose concentrations and repressed in low glucose concentrations. HXT7 is a high affinity glucose transporter expressed in low glucose concentrations and repressed in high glucose concentrations.

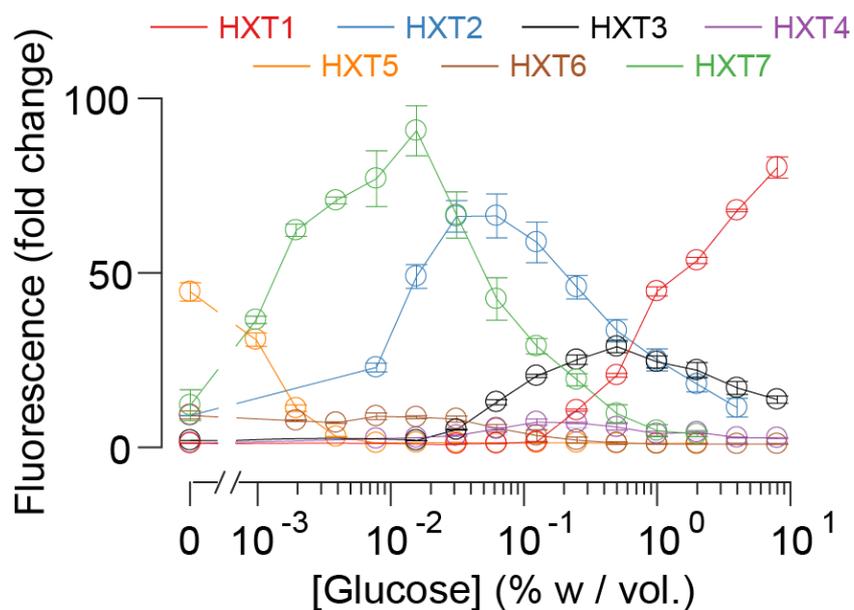


Figure 44 Expression level of glucose transporters HXT1-7 in batch culture measured as fluorescence fold change (normalized by the negative control expression) as a function of different glucose concentrations. X-axis is in log<sub>2</sub> scale.

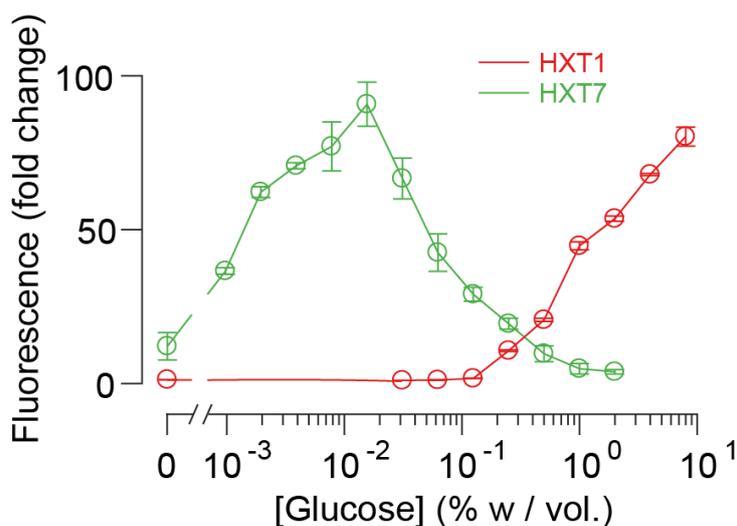


Figure 45 Comparison between two glucose transporters that have an opposite expression pattern. HXT1 is a low affinity transporter that is expressed in high glucose concentrations and HXT7 is a high affinity transporter that is expressed in low glucose concentrations. X-axis is in log<sub>2</sub> scale.

Knowing the gene expression landscape of HXT1-7 in batch cultures gave us an approximate idea of what to expect in colonies. We then studied the same genes (HXT1-7) in the microfluidic devices. Cells were loaded in the “Yeast Machine”, centrifuged into cell chambers, and grown for 10 hours as a full colony on the chip before we measured the expression level of glucose transporters at low

magnification (10x). In all conditions, we observed a non-homogeneous level of expression and the emergence of a landscape of gene expression. Looking at all seven transporters in a standard 2% (111 mM) glucose concentration media we can see that each of the transporters is differentially expressed at different positions within a colony (Figure 46). Even if we would compare expectations from the gene expression in batch with the expression in a colony, we would notice that it is very consistent. HXT1 is expressed closest to the nutrient source, followed by HXT3 which is expressed in a wider range of glucose concentrations, HXT4 and HXT2 are expressed next, HXT6 and HXT7 have a peak of expression in low glucose concentration, and at the back of the colony HXT5 is expressed which expression is induced by entering into growth arrest and lack of glucose. This is in agreement with the fact that glucose is progressively absorbed by cells, thus creating a spatially evolving microenvironment to which cells adjust. Describing quantitatively such maps of gene expression is important to better understand and model the behaviour of multicellular yeast assemblies and to understand their local microenvironments. It is even more interesting if we consider that the experimental conditions are rather simple and that the observed gene expression landscape emerges from the collective behaviour of cells and, what we call, long range metabolic interactions.

We decided to use a heatmap for most of our gene expression data so that we can easily represent a three-dimensional space of gene expression, position inside a colony, and glucose concentration (or the type of gene). While this kind of representation gives more clarity, it is hard to represent the variability of each experiment. This is why at the end of this thesis you can find Additional figures where all the data from the heatmaps has been pooled together in one graph with included standard deviation represented by the shaded error bar. The heatmaps were produced from the means of that data. Another thing worth noticing is that although the “Yeast Machine” cell chamber is about 800  $\mu\text{m}$  long we cut off our data at 700  $\mu\text{m}$ . The reason for this is that cells do not fill out the chamber completely but there is a gap between the nutrient channel and the cell chamber because cells are washed away by the flow. The cut at 700  $\mu\text{m}$  distance from the nutrient source ( $z$  ( $C = C_0$ ) = 0  $\mu\text{m}$ ) is set at the colony front, not at the junction of the nutrient channel and cell chamber because  $C_0$  is located at the colony front) is for representation purposes only, so that the lines do not slightly vary in size as the colony front is not always at the same position across all the experiments. Data in the Additional figures are plotted with the complete distance ( $z > 700\mu\text{m}$ ) where it is possible.

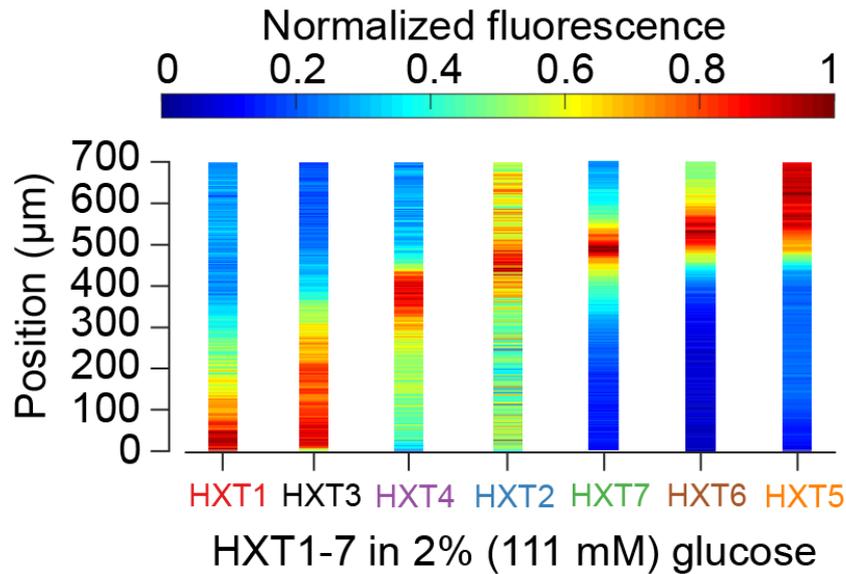


Figure 46 Gene expression pattern of glucose transporters HXT1-7 in 2% glucose media. Expression of each gene was normalized by the maximum of its expression. Position 0  $\mu\text{m}$  is the position of the glucose source. Each gene is expressed at a different position inside a colony. Additional figure can be found here (Figure 99).

## Landscape of gene expression depends on the glucose source concentration

We demonstrated that there is a landscape of gene expression of different genes in a standard 2% glucose concentration. I will turn the focus now on the role of the starting glucose concentrations. Increasing the source glucose concentration in the nutrient channel led to a change of the landscape of gene expression of all hexose transporters. We were especially interested to see what happens with the two glucose transporters which have such stark, almost completely opposite differences in the expression pattern – HXT1 and HXT7. At 1% (55.5 mM) glucose concentration HXT1 is only slightly expressed at the beginning of the colony ( $z < 60 \mu\text{m}$ ), but at the highest glucose concentration that we used (8%; 444 mM), HXT1 is expressed at high levels throughout the whole colony, except at the very back when it starts to decay (Figure 47). In between those two extreme concentrations, HXT1 expression gradually penetrates deeper into the colony and its levels become higher, in line with our hypothesis of glucose gradient formation within a colony. In batch conditions HXT1 is expressed in high glucose conditions, this experiment shows that the glucose penetration is increasing with the increase in external glucose concentration.

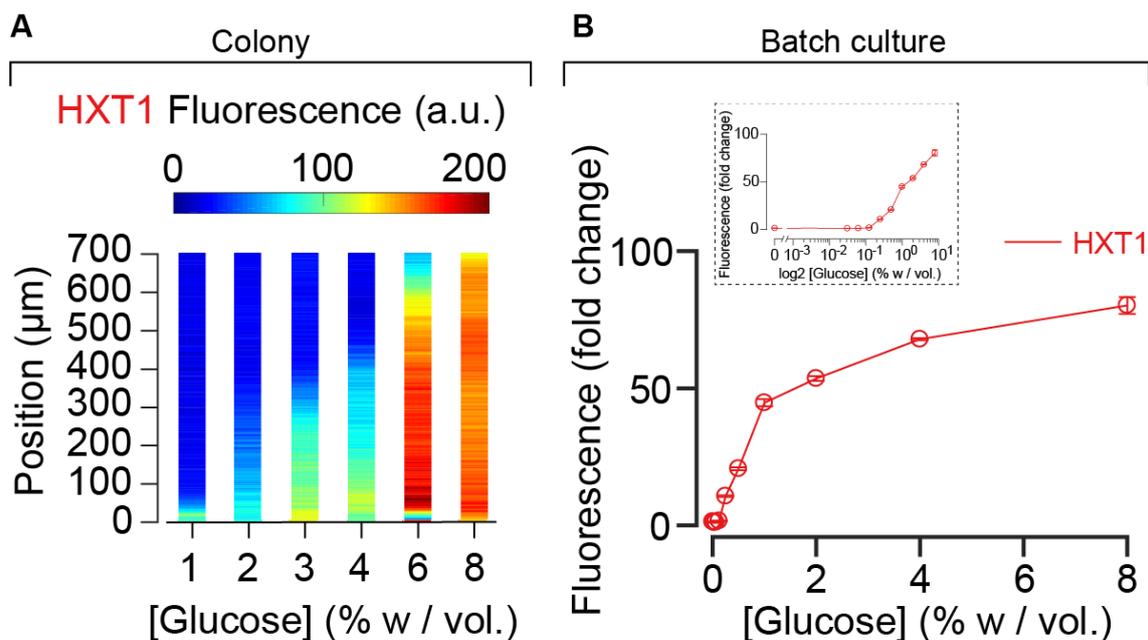


Figure 47 Gene expression landscape of the glucose transporter HXT1 in different glucose concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, HXT1 is expressed ever deeper and stronger as starting glucose concentration is increased. Fluorescence values represent measured values after the background is removed. (B) In batch cultures, HXT1 is expressed in high glucose concentrations (main figure: linear x-axis; insert:  $\log_2$  x-axis). Additional figure can be found here (Figure 100).

HXT7, on the other hand, exhibits a peak like expression pattern, it is repressed both in high glucose and when there is no glucose present. At very low glucose concentrations 0.01% (0.55 mM) we observed a peak right at the beginning of a colony ( $z \sim 10 \mu\text{m}$ ), indicating that glucose is quickly absorbed by cells (Figure 48). The peak moves deeper into the colony as the glucose concentration at the source is increased and disappears completely in 8% (444 mM) glucose concentration, indicating that there is glucose everywhere in the channel (Figure 49). The peak of maximum expression doesn't increase linearly with the increase of glucose concentration. As glucose is increased it seem to slow down. This probably underlies a complex interplay between diffusion, glucose uptake rate, and starting glucose concentration, which produces glucose gradients inside a colony. The landscape of gene expression of HXT1 and HXT7 are thus in good agreement with a description in which cells self-generate a glucose gradient which properties depend on the interaction between cells and their environment, namely external glucose conditions.

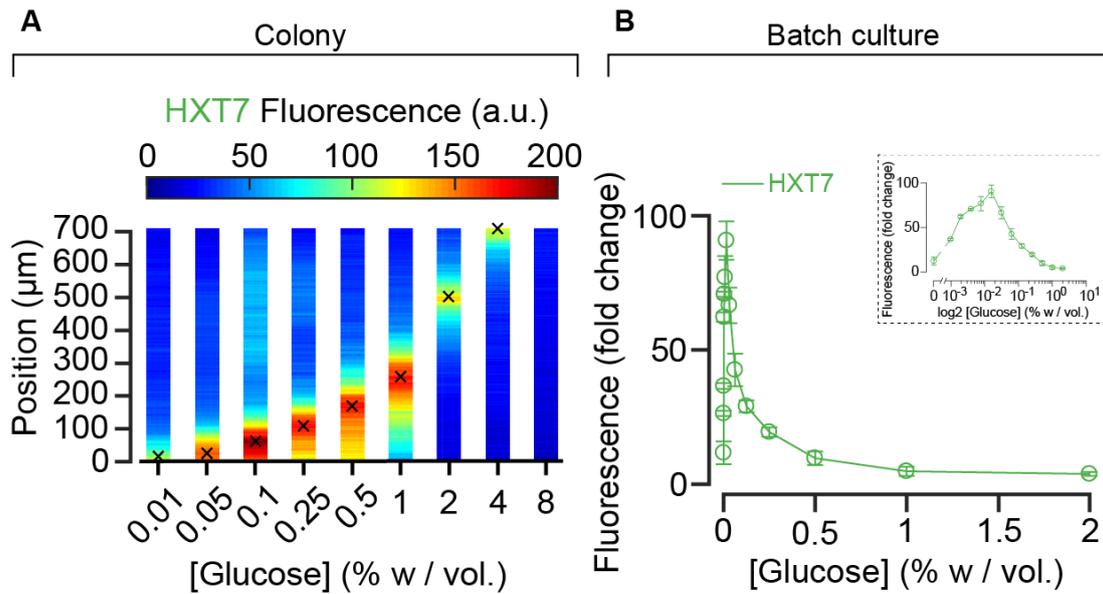


Figure 48 Gene expression landscape of the glucose transporter HXT7 in different glucose concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, the peak like expression pattern moves deeper inside colony as starting glucose concentration is increased. Black x represents the peak of expression. Fluorescence values represent measured values after the background is removed. (B) In batch cultures, HXT7 has a peak of expression in low glucose concentrations (main figure: linear x-axis; insert:  $\log_2$  x-axis). Additional figure can be found here (Figure 101).

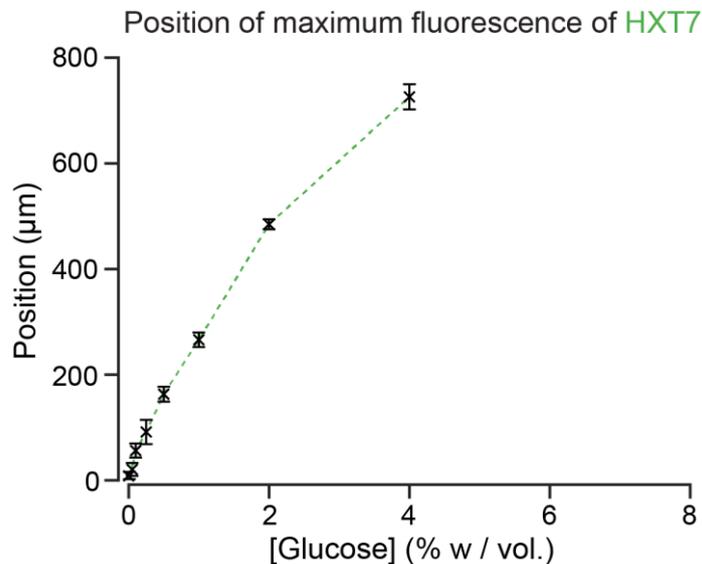


Figure 49 Peak of expression of glucose transporter HXT7. As starting glucose concentration increases, the peak of maximum expression moves deeper into the colony. The position of the peak doesn't move linearly with the increase of starting glucose concentration, but it seems like to slow down as glucose is increased.

Research on a topic like this is very visual and involves thousands of hours of movies. Unfortunately, we still do not have technology to embed videos on a piece

of paper but we can make kymographs, snippets of our colonies in time and beautiful patterns that they make. When cells that have HXT7 endogenously tagged with GFP are loaded in the cell chamber they express almost no fluorescence (Figure 50 A, C). But at one point when glucose concentration becomes sufficiently low, cells start to express HXT7 and pattern of expression appears. As colony grows, HXT7 pattern shifts depending on the size of the colony, until the colony reaches its definitive size in the cell chamber and the pattern takes a more constant form. The same principle is true for HXT1 endogenously tagged with GFP (Figure 50 B, D). Cells are loaded in the cell chamber and they do show some fluorescence because of the culturing conditions before the experiment. As colony expands cells quickly start to express HXT1 because of high glucose concentration 2% (111 mM) and 4% (222 mM), until glucose becomes too low locally in the colony and expression is shut down.

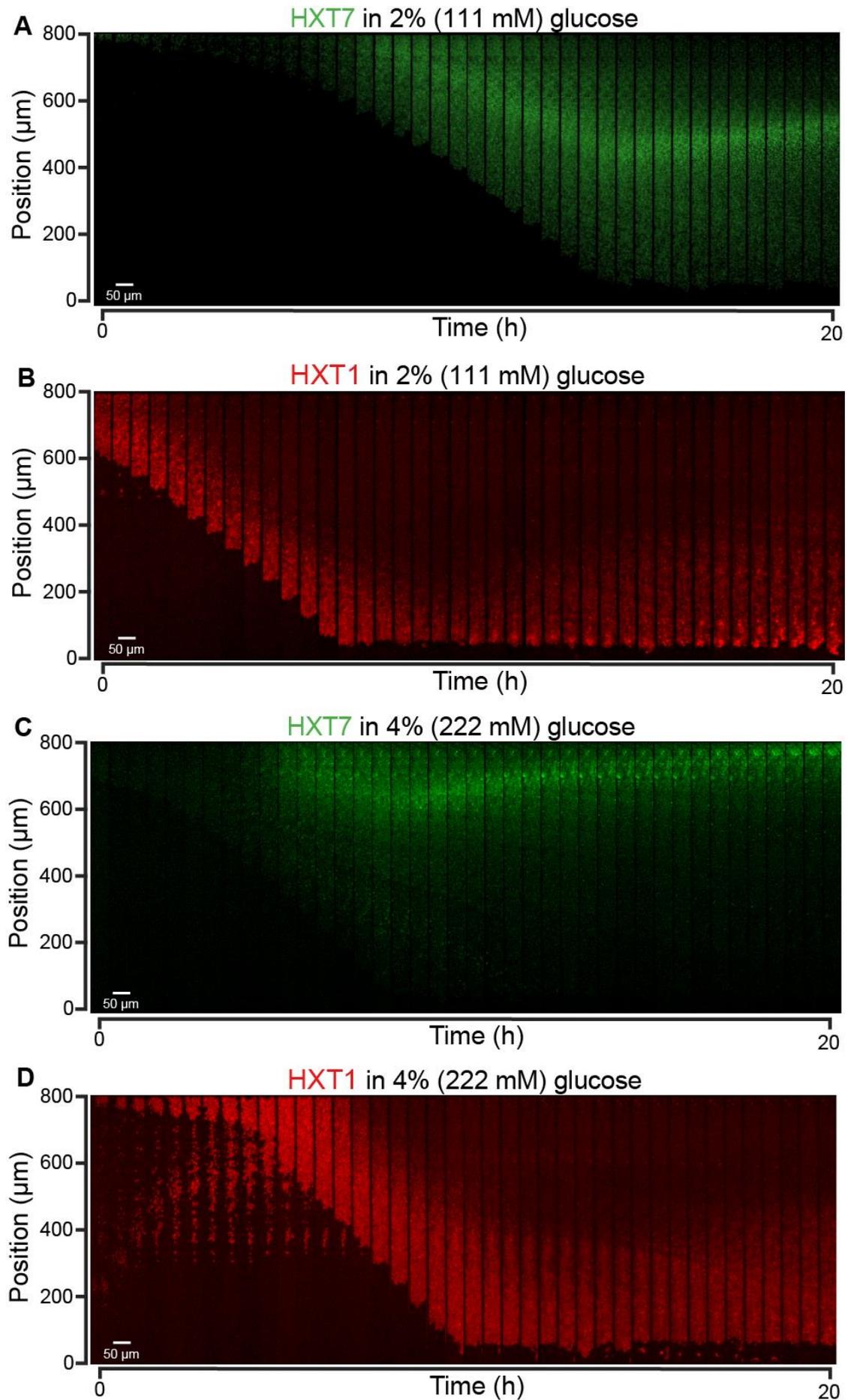


Figure 50 (previous page) Kymograph of glucose transporter HXT7 in 2% glucose and HXT1 in 4% glucose. Time step between two images of the same colony is 30 minutes. Position 0  $\mu\text{m}$  in this case reflects the beginning of the cell chamber and not the starting glucose concentration as one might observe that the edge of the colony is not at the same position as the cell chamber to nutrient channel junction. Time 0 h is time when the cells were loaded and the number of loaded cells is not always constant. (A, C) As HXT7 labelled colony is expanding, a pattern of expression starts to appear in area of low glucose. (B, D) As HXT1 labelled colony is expanding, a pattern of expression starts to appear in area of high glucose.

Let us take a closer look inside a colony that has GFP tagged glucose transporter HXT7. If we divide the colony in three parts, one close to the nutrient source, second at the peak, and third at the back of the colony, we can see differences between cells (Figure 51). Around the peak all cells express HXT7 and Hxt7p is accumulated on the membrane in certain number. But what happens downstream and upstream of that? Closer to the nutrient source cells grow, divide, and are constantly pushed through the glucose gradient at some velocity. This leads to a constant adaptation to ever changing environments as well as dilution through division. On top of that, it is known that HXT7 transcription is repressed and Hxt7p is targeted for degradation in the vacuole in response to glucose abundance, nitrogen starvation, and rapamycin treatment [195]–[198]. On the opposite side, far away from the nutrient source, cells do not divide so proteins cannot be diluted, but Hxt7p still is ubiquitinated, targeted for endocytosis and degraded in the vacuole. It is a different mechanism than the one involved in response to high glucose concentrations. The response of Hxt7p to glucose deprivation has recently been discovered in Sébastien Léon's group and thoroughly characterized [199]. Upon transfer to a glucose deprived media cells trigger endocytosis of Hxt7p via ubiquitylation of ART protein Csr2/Art8, which leads to a subsequent accumulation in the vacuole and degradation (Figure 52). In a mutant *csr2-1* strain endocytosis is not activated so Hxt7p stays localized on the membrane regardless of glucose deprived environment.

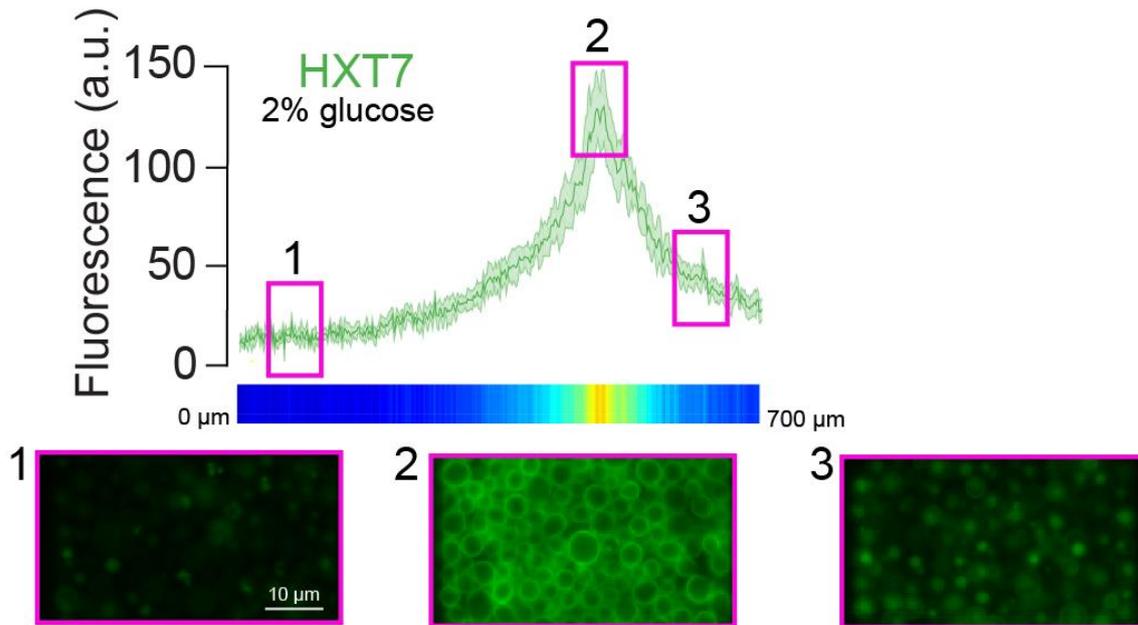


Figure 51 Close up of three different positions inside a colony. Position 0  $\mu\text{m}$  is the position of the glucose source. Glucose transporter HXT7 peaks at 491  $\mu\text{m}$  distance from the glucose source. At this position almost all GFP is located on the membrane. At one point in glucose concentration gradient Hxt7p is ubiquitinated, targeted for endocytosis and degraded in the vacuole. This happens either when glucose concentration is almost near zero (panel 3) or when glucose concentration is high (panel 1). In near zero and zero glucose conditions cells basically do not divide, so fluorescence is not diluted. On the other hand, when glucose is present cells divide and dilute fluorescence this way too in addition to targeted degradation.

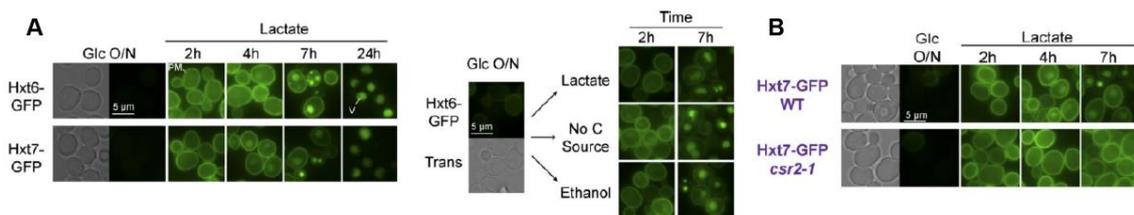


Figure 52 Regulation of endocytosis of Hxt6p and Hxt7p upon glucose deprivation. Figure adapted from [199]. (A) In glucose rich conditions HXT6 and HXT7 are not expressed. Upon transfer into a glucose deprived media, ART protein Csr2/Art8 is activated and targets Hxt6 and Hxt7 for endocytosis. (B) In *csr2-1* mutant cells, endocytosis of Hxt7p is not triggered and it stays localized on the membrane.

Léon's group also checked this effect in HXT6 GFP-tagged colonies grown on an agar plate for 4 days by cutting the colony vertically down the middle and then imaged with two-photon confocal microscopy [199]. In *csr2-1* mutant, fluorescence intensity was higher and the spread of cells that carry Hxt6p on the membrane was wider across colony in comparison with the WT cells. Also, cells far away from the nutrient source (at the top of the colony) exhibited Hxt6p localization on the membrane (although at lower levels than the cells closer to the nutrient source), whereas in WT cells fluorescence was localized in the vacuole. We were kindly provided with the WT and mutant cells from Léon's group so that we can test them in our system (Figure 53). One needs to be careful with the interpretation of the results between the two experiments, original in colonies on an agar plate and our test in the "Yeast Machine", for at least three reasons: 1) we looked at HXT7 and they looked at HXT6 (although they are both high-affinity transporters similar to each other when expressed in a colony), 2) we used standard well-defined media with 2% glucose and they used glycerol-ethanol complete respiratory medium (3% glycerol, 1% ethanol), and 3) colonies are not of the same size (our colony is ~ 800  $\mu\text{m}$  high and their colony is ~ 300  $\mu\text{m}$  high) and the imaging did not happen at the same point in time. Nevertheless, many points are comparable between the two. The peak of expression is higher in mutant cells, the expression is spread wider, and at the end of the colony, in areas where glucose should be depleted, expression levels are significantly higher, indicating that mutant cells preserved Hxt7p on the membrane.

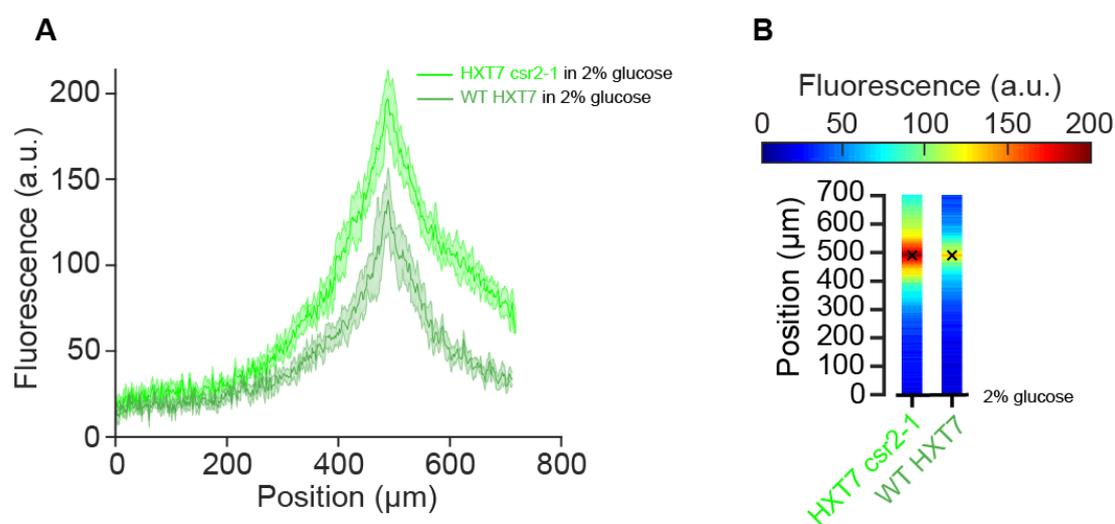


Figure 53 WT HXT7 and HXT7 *csr2-1* mutant expression in 2% (111 mM) glucose in the "Yeast Machine". (A) Comparison between the two expression profiles. (B) Same results from a different view. Black x indicates the peak of expression. Position 0  $\mu\text{m}$  is the position of glucose source.

I will finish this chapter with the comparison between the two glucose transporters. HXT1 is a low-affinity transporter expressed in high glucose concentrations and HXT7 is a high-affinity transporter expressed in low glucose concentrations. If we assume that there is a formation of glucose concentration gradient as a consequence of non-trivial interactions between diffusion, glucose uptake, and starting glucose concentrations, together with dependence on local microenvironments and adaption of cells that move through them, we should see differentiation of genes which expression depends on specific environments. As HXT1 and HXT7 are such genes and their expression pattern in well mixed homogenous environments is exactly the opposite of each other, they were a good choice to look at. Even more so because they are the genes that dictate one of the most important elements in the formation of glucose gradients, namely the uptake rate. As it is obvious, **these two genes are expressed at different positions in the colony resulting in differentiation within a colony and self-organization of isogenic single cells exhibiting emergent properties which they could not on their own** (Figure 54).

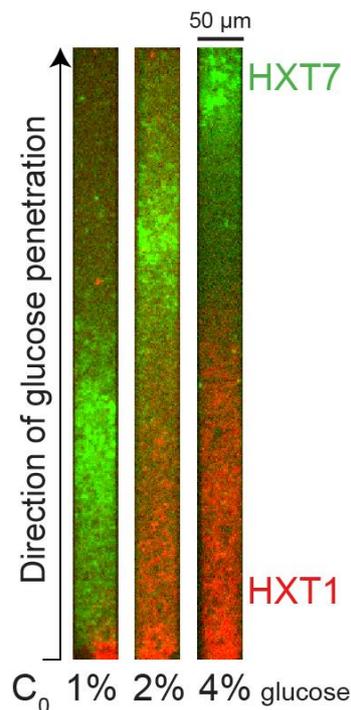


Figure 54 Merged images of gene expression of HXT1 (red) and HXT7 (green) in high glucose concentrations. HXT1 and HXT7 have opposite expression pattern. As glucose is increased HXT1 is expressed ever deeper in the colony, where ever there is sufficiently high glucose. HXT7 expression peaks in low glucose concentrations, this peak of expression moves deeper in the colony as glucose is increased. We can clearly observe differentiation of two subpopulations that express HXT1 in high glucose concentrations and HXT7 in low glucose concentrations.

## **Reconstruction of the glucose gradient in yeast colonies**

In the course of this project, from the PhD selection committee and afterwards, I have heard several times that it will be almost impossible to measure the environment inside colony, especially the glucose concentration. We have carefully considered all the possible options that we could do, with the most prominent one being the use of some kind of sensors. The main focus of glucose sensor development is the efficacy of measuring blood glucose, and in this field many different options have been developed [200]. However, a large majority of such sensors is sensitive to low glucose concentrations ( $\mu\text{M}$  to low  $\text{mM}$  range) and not high concentrations that we usually use (up to 444  $\text{mM}$ ). Even sensors that might sense high enough concentrations are either not possible to adapt to our microfluidic device or they are very sensitive to the environmental conditions which would probably render them unusable in our colonies with local environmental heterogeneities. This is why we decided to put to use the HXT glucose transporters in one more task, apart from gene expression differentiation. We wanted to measure if cells themselves could report to us the glucose concentration in their microenvironment. The case of HXT7 is particularly interesting for this task since its landscape of expression allows us to define a distance at which the glucose concentration is around 0.0156% (0.86  $\text{mM}$ ), measured as the peak of expression. Assuming that the level of expression of HXT7 is set by the local glucose concentration, we can use the batch culture expression data to determine the glucose concentration at a given position, based on the HXT7 level of expression in batch culture and within a colony. Moreover, we could apply the same logic by using the landscape of gene expression of other HXTs, in particular HXT1. As we demonstrated previously, HXT1 and HXT7 have an opposite expression pattern so their “dynamic range” of expression in different glucose concentrations is complementary. This could be used to improve and get a better resolution of the glucose concentration gradient inference and allow us to reconstruct the glucose concentration profile within the yeast assembly that we studied at different starting glucose concentrations. Hence, we used glucose transporter HXT7 to reconstruct glucose gradients in starting glucose concentrations ranging from 0.1% (5.5  $\text{mM}$ ) to 4% (222  $\text{mM}$ ). We also used HXT1 in 2% and 4% starting glucose concentration to

increase the resolution of reconstruction and to see how it compares to the reconstruction with HXT7 (Figure 55).

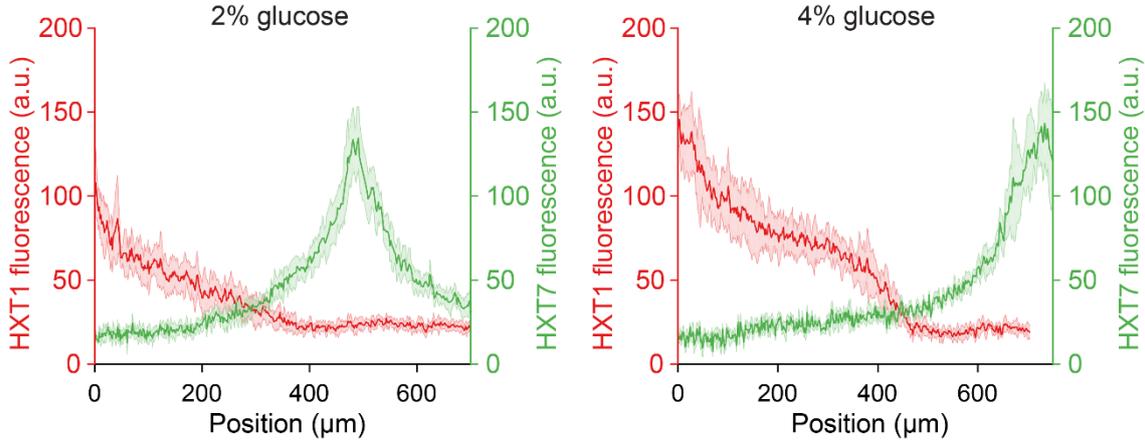


Figure 55 Gene expression of glucose transporters HXT1 and HXT7 in 2% and 4% starting glucose concentration. HXT7 has a wide “dynamic range” deeper in the colony where glucose is low, and HXT1 has a wider “dynamic range” in high glucose close to the nutrient source.

We used the following method to infer glucose concentration gradient as a function of position. We know two fixed in colony and in bulk culture. These points are fluorescence intensity at starting glucose concentration  $F_0$  and point of maximum fluorescence intensity  $F_{max}$  in case of HXT7 or point of minimum fluorescence intensity  $F_{min}$  in case of HXT1 (Figure 56). Let us take an example of 2% starting glucose concentration for HXT7 expression. In colony,  $F_0$  is located at position  $0 \mu\text{m}$  and  $F_{max}$  is located at position  $491 \mu\text{m}$ . In bulk culture,  $F'_0$  corresponds to 2% glucose and  $F'_{max}$  corresponds to 0.0156% glucose. Hence, at position  $0 \mu\text{m}$  there is 2% glucose and at position  $491 \mu\text{m}$  there is 0.0156% glucose. Now we need to infer the corresponding points for each position and each glucose concentration by using information about the level of gene expression. We do this with following equations:

$$\frac{F_n - F_0}{F_n - F_{max}} = \frac{F'_n - F'_0}{F'_n - F'_{max}} \quad \text{Eq. 17}$$

$$\alpha = \frac{F'_n - F'_0}{F'_n - F'_{max}} \quad \text{Eq. 18}$$

$$F_n = \frac{F_0 - \alpha F_{max}}{1 - \alpha} \quad \text{Eq. 19}$$

Where,  $F_0$  is the fluorescence intensity at the position of starting glucose concentration in colony,  $F'_0$  is the fluorescence intensity in the corresponding starting glucose concentration in bulk culture,  $F_{max}$  is the maximum fluorescence

intensity in colony,  $F'_{\max}$  is the maximum fluorescence intensity in bulk culture,  $F_n$  is any fluorescence intensity between  $F_0$  and  $F_{\max}$ ,  $F'_n$  is any fluorescence intensity between  $F'_0$  and  $F'_{\max}$ , and  $\alpha$  is a factor that correlates colony and batch fluorescence values. This means that for each  $F'_n$  in glucose concentration  $C_n$  we can infer the corresponding  $F_n$  at position  $X_n$  and reconstruct the glucose concentration gradient as a function of position within colony. The procedure is similar for HXT1, except that we know the position and concentration of minimum fluorescence. Each curve was fitted to get a monotonic increase or decrease of fluorescence intensity.

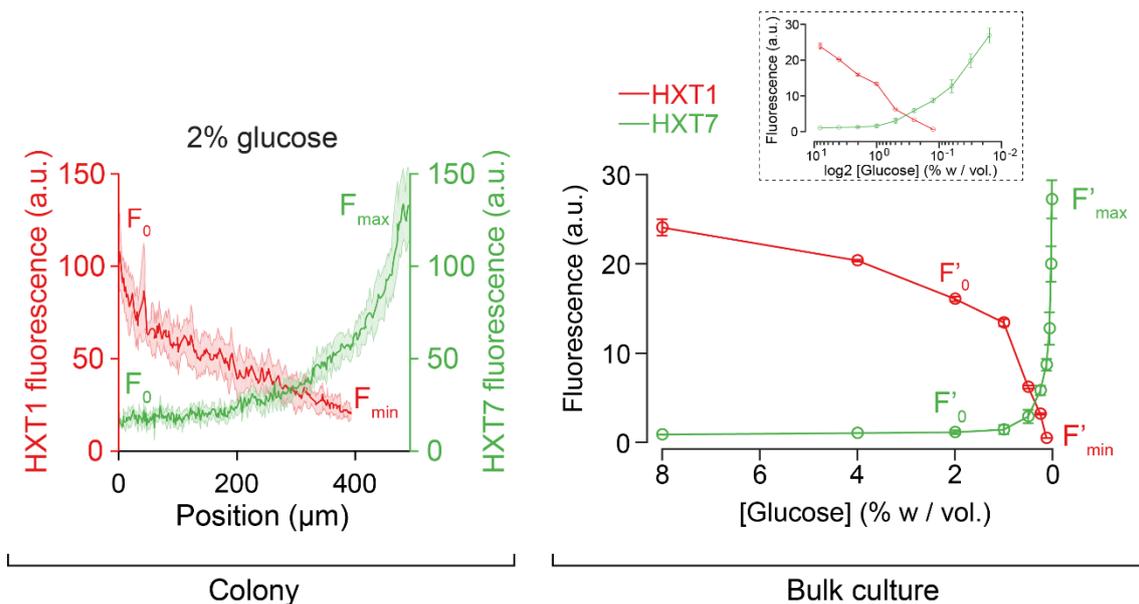


Figure 56 Gene expression of glucose transporters HXT1 and HXT7 in 2% starting glucose concentration in colony (left), and in all glucose concentrations in bulk culture (right). Bulk culture insert has a  $\log_2$  x-axis. There are two fixed points in expression pattern, fluorescence intensity at starting glucose concentration  $F_0$  and point of maximum fluorescence intensity  $F_{\max}$  in case of HXT7 or point of minimum fluorescence intensity  $F_{\min}$  in case of HXT1. By combining expression levels in colony and in bulk we can infer glucose concentration at each position in the colony. For more details see main text.

Judging by the reconstructed glucose concentration gradients within colony, glucose is not depleted in a linear fashion. In higher glucose concentrations, closer to the nutrient source glucose seems to be depleted faster, and then this depletion slows down (Figure 57). This might make sense, as we know that glucose transporters with lower affinity but higher maximum uptake rates are expressed in higher glucose concentrations. This makes them less prone to saturation by glucose and it is possible that they deplete glucose much faster from the environment. We can also see that concentration gradients obtained by HXT1 and HXT7 overlap in most parts, indicating that this method might be consistent and robust. Although, we are not claiming that the obtained glucose concentrations represent the real

concentrations within a colony as the gene expression intensity might have been “filtered” through different interactions between environment, phenotype, genotype, growth, and adaptation. Sadly, *Star Trek* like tricorders still do not exist. But hopefully, in the near future new physico-chemical methods will be developed which might be used to decipher all different molecules within the environment of a colony. In addition to this, it is interesting to think about how evolution might have influenced the optimization of nutrient uptake system in a way that the yield of the colony is maximized. From a colony perspective, it would make sense to increase the availability of nutrients to as much as possible constituents of the colony. One way to do it is to optimise glucose uptake and growth rates that will lead to higher yields. Even if evolution did not act on a colony level, it is interesting to think about this problem from the perspective of synthetic biology and colony/microbiome/community engineering. Resource reallocation optimization strategies on a single cell level, from a fundamental and engineering perspective, have been discussed by Geiselman and de Jong here [201], [202].

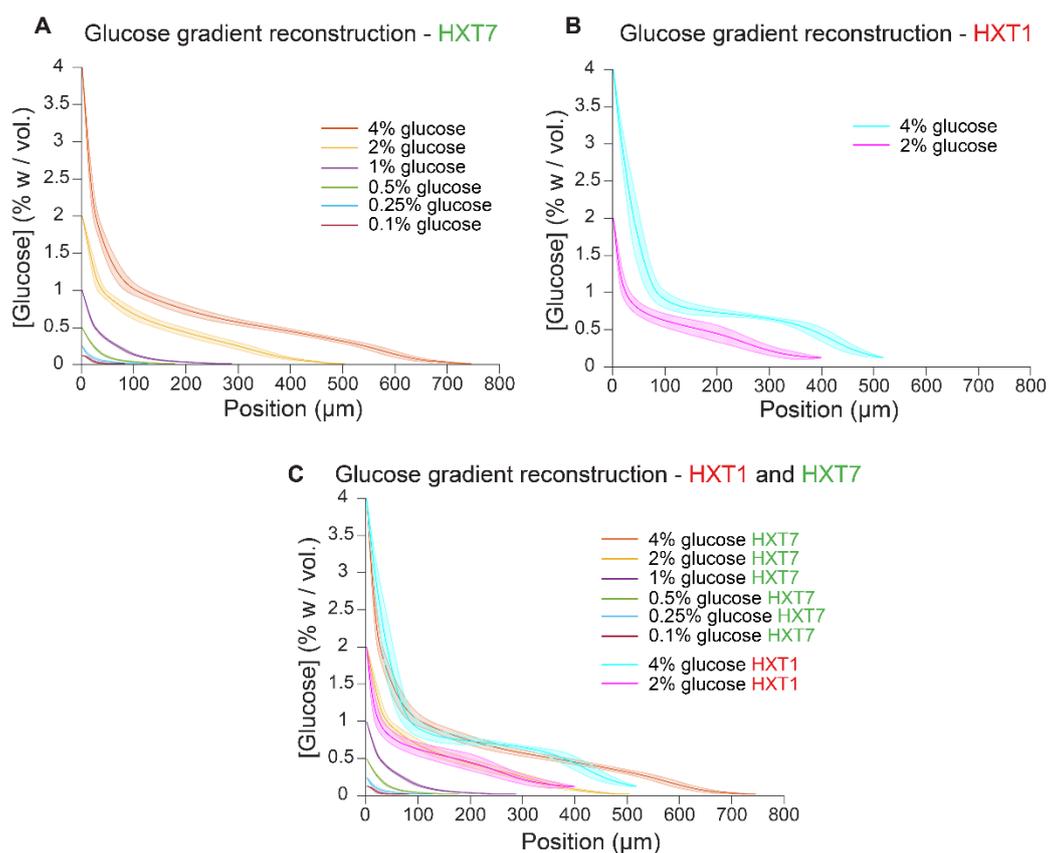


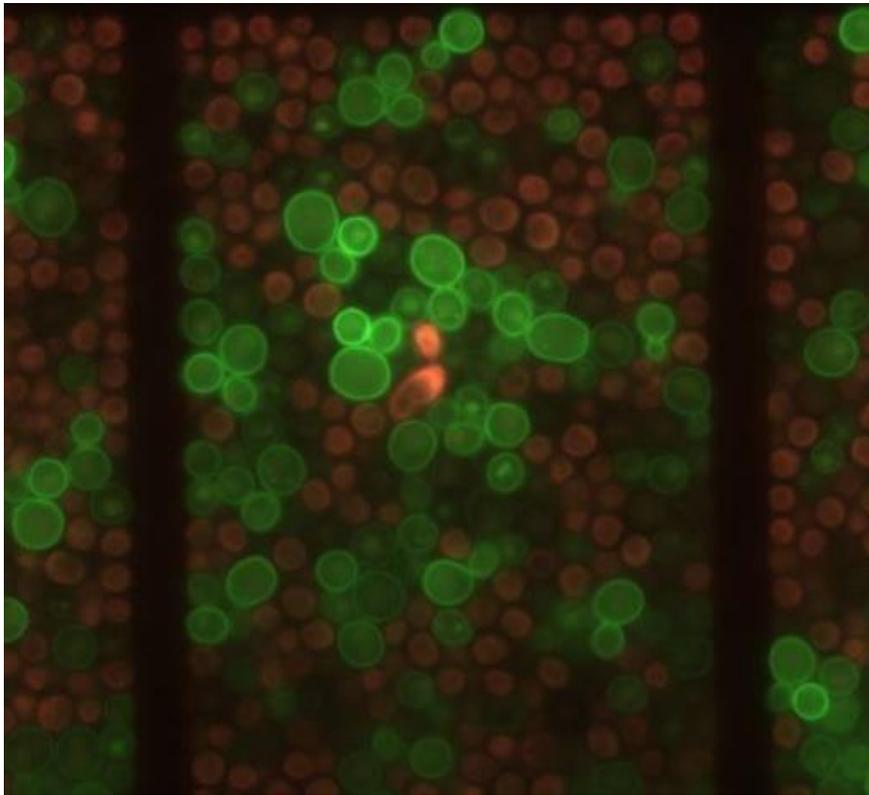
Figure 57 Reconstruction of the glucose concentration gradient within *S. cerevisiae* colony. (A) Reconstruction based on glucose transporter HXT7 expression. (B) Reconstruction based on glucose transporter HXT1 expression. (C) Combined reconstructed gradients based on HXT1 and HXT7 expression. Position 0  $\mu\text{m}$  is the position of glucose source. Each glucose concentration indicates the starting glucose concentration.

In Chapter II we demonstrated how quickly and easily phenotypic differentiation emerges when cells grow together and form heterogeneous environments. We also showed that different major glucose transporters are expressed at different positions within colonies, each one of them responsible for setting the glucose uptake at specific parts of the colony. We took the example of transporters HXT1 and HXT7 which have the opposite expression pattern and demonstrated their differentiation in population in high glucose concentrations (HXT1) and in low glucose concentrations (HXT7). We further pushed this exploit of glucose transporters and decided to use them as reports for local glucose concentrations.

Now we wonder if other genes also follow this established pattern of expression that depends on glucose concentration, so in next chapter I will present several phenomena that are tightly connected to glucose concentrations in the environment.



## **Chapter III: Growth arrest and glucose metabolism in yeast colonies**





Glucose transporters are not the only genes that can inform us of the metabolic landscape of a colony. As a matter of fact, in a perfect world we would be able to characterize all of ~6000 genes of yeast *S. cerevisiae* and map their expression in any environment of our choice. To do this in microfluidics, we would need technologies that do not exist yet or that are still in the prototyping phase. With the high number of genes and many imaginable starting environments, the combination of possible experiments is staggering. Even more so if we include gene knock-outs into the space of possible combinations. However, it is not hard to imagine that one day we will be able to load tens or hundreds of strains independently from one another and be able to feed a multiplex of environments just on one chip. But for now, we have to stick to our chip onto which we can load two different strains and feed two different environments independently. As I previously showed, with this we can observe how colonies behave in the full glucose concentration gradient, from the starting concentration of our choice to a complete depletion. This fact also makes using the “Yeast Machine” a convenient method to look at how cells respond in the whole possible concentration gradient of any nutrient.

We chose to take a closer look at several different phenomena that are tightly connected to glucose metabolism. We mapped the landscape of growth arrest across a range of starting glucose concentrations, we looked at glucose repression and how it evolves in the glucose concentration gradient, we studied how colonies differentiate into subpopulations that ferment and subpopulations that respire, and finally we took a glimpse into how glucose gradients affect glycolysis inside colonies. At the end I will summarize everything presented so far to see if all these phenomena behave consistently within colonies or they diverge beyond reasonable assumptions that we could make looking at their features in well mixed liquid cultures made of homogeneous environments.

## **Growth arrest in the structured environment of a yeast colony**

Glucose transporter HXT5 is a very convenient gene to study since it is expressed when cells enter the growth arrest. By using this gene, we can nicely map cells that are growing extremely slow or not growing at all. In low glucose concentrations of 0.1% (55.5 mM) HXT5 is not expressed in the parts of the colony closes to the glucose source, but it is expressed deeper in the colony. As we increase starting glucose concentrations the parts of the colony that do express HXT5 shrink ever more and then in the high glucose concentrations of 4% (222 mM) only a short band of cells that are the furthest from the glucose source start to express HXT5, meaning that most of the cells in the colony grow and do not enter in the state of growth arrest (Figure 58 A). In batch cultures, no HXT5 is expressed in high glucose concentrations, but rather the expression is induced in the area of very low glucose concentration, somewhere between 0.01% (0.55 mM) and 0.001% (0.055 mM), and it reaches its maximum when no glucose is present in the media (Figure 58 B). To quantify the position of expression better, we fitted a sigmoidal curve to our data since the expression follows a sigmoidal pattern, HXT5 is off or on and between these states there is a quick transition. The sigmoidal pattern appears in the batch culture too. By fitting a sigmoidal curve, we could calculate the position of half maximum expression, in between the “on” and “off” states (Figure 59). This position should also correspond to the area of low glucose concentration, close to 0.001% (0.055 mM).

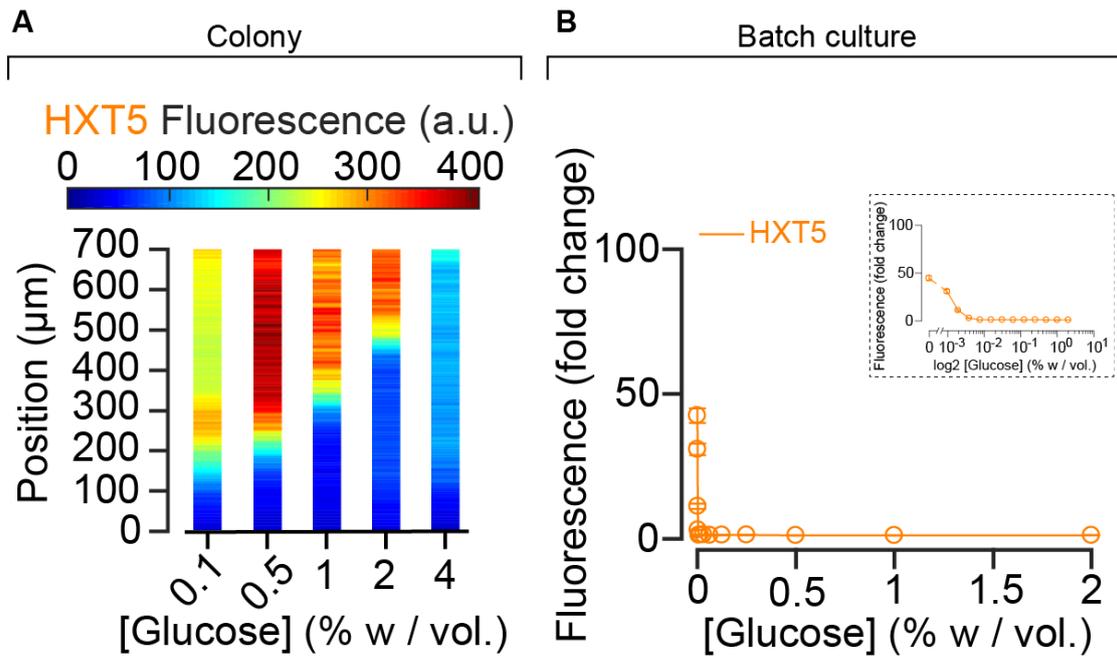


Figure 58 Gene expression landscape of the glucose transporter HXT5 in different glucose concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, cells that are growing in the presence of glucose do not express HXT5, while cells that do express HXT5 are in a growth arrest. Fluorescence values represent measured values after the background is removed. (B) In batch cultures, HXT5 is not expressed in high glucose concentrations, but rather the expression is triggered in very low glucose concentrations and in the absence of glucose (main figure: linear x-axis; insert: log<sub>2</sub> x-axis). Additional figure can be found here (Figure 102).

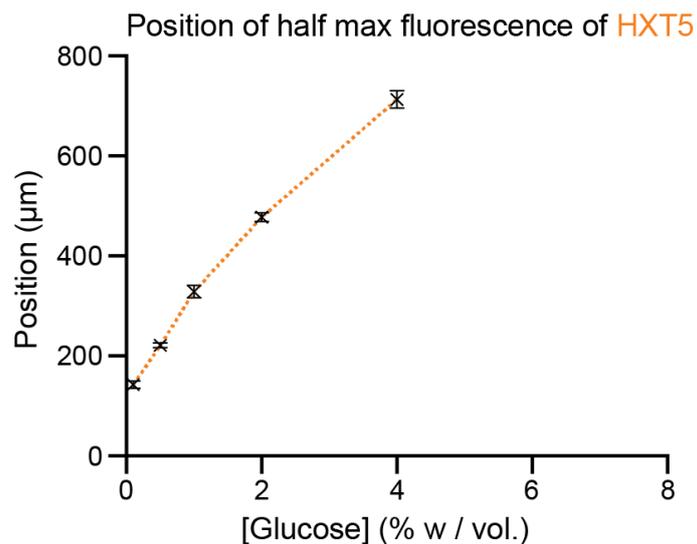


Figure 59 Position of the half maximum fluorescence of the glucose transporter HXT5. As starting glucose concentration increases, the half maximum expression moves deeper into the colony.

We will now take a closer look at what is happening in the colony from the point when just a couple of dozens of cells are in the cell chamber to the time when a full colony is formed. When cells that have HXT5 endogenously tagged with GFP are loaded in the cell chamber they express almost no fluorescence (Figure 60). But at the point when glucose concentration becomes sufficiently low, cells start to express HXT5 and pattern of expression appears. As colony grows, HXT5 pattern gets larger depending on the size of the colony, until the colony reaches its definitive size in the cell chamber and the pattern takes a more constant form. As the starting glucose concentration increases, the area of expression decreases. The interaction between the colony length and starting glucose concentration is also visible, since the pattern of expression will start to appear only when glucose becomes sufficiently low which depends on the colony size and the starting glucose concentration.

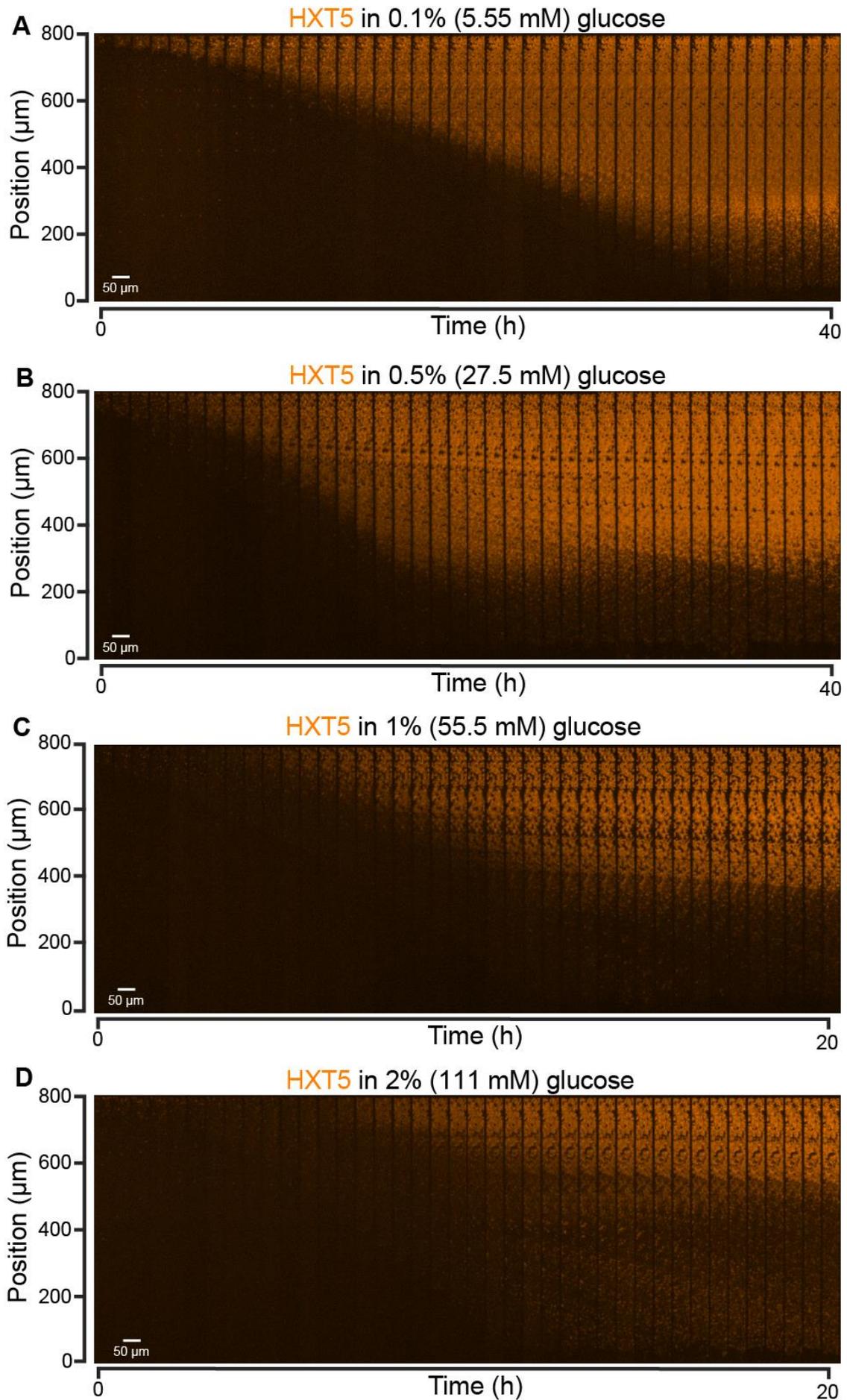


Figure 60 (previous page) Kymograph of glucose transporter HXT5 in (A) 0.1%, (B) 0.5%, (C) 1%, and (D) 2% starting glucose concentration. Time step between two images of the same colony is 60 minutes for 0.1% and 0.5% glucose, and 30 minutes for 1% and 2% glucose. Position 0  $\mu\text{m}$  in this case reflects the beginning of the cell chamber and not the starting glucose concentration as one might observe that the edge of the colony is not at the same position as the cell chamber to nutrient channel junction. Time 0 h is time when the cells were loaded and the number of loaded cells is not always constant. As HXT5 labelled colony is expanding, a pattern of expression starts to appear in the area of very low glucose concentrations and where there is no glucose. Size of the colony in interaction with different starting glucose concentrations, determines where the expression will start. The area where HXT5 is expressed also depends on the glucose concentration.

If we divide the colony in three parts, one close to the nutrient source, second at the transition phase, and third at the back of the colony, we can see differences between single cells (Figure 61). In the parts of the colony where glucose is deprived Hxt5p accumulates on the membrane. Close to the glucose source we can observe almost no fluorescence and cells have mostly diluted, degraded or shut down the expression of HXT5. In the transition phase there is still some Hxt5p localized on the membrane, but it is also targeted for degradation in the vacuole through the process of endocytosis. While it is known that HXT5 gene transcription seems to be under the control of two stress-responsive elements (STREs), two Hap2/3/4/5p (HAP) complex binding elements, and one post-diauxic shift (PDS) element in its promoter region [203], not much is known about its degradation, unlike for HXT7. Researchers looked at the degradation mechanisms and concluded that the addition of glucose to stationary-phase cells resulted in a transient phosphorylation of Hxt5p on serine residues, however they did not detect any ubiquitylation [204]. They did see internalization and degradation of Hxt5p in the vacuole, so it seems like Hxt5p is degraded via endocytosis pathway like other glucose transporters, but it is independent of ubiquitylation. Léon's group discovered that endocytosis of Hxt5p did not require Csr2, a protein involved in the endocytosis process that is activated by ubiquitylation [199].

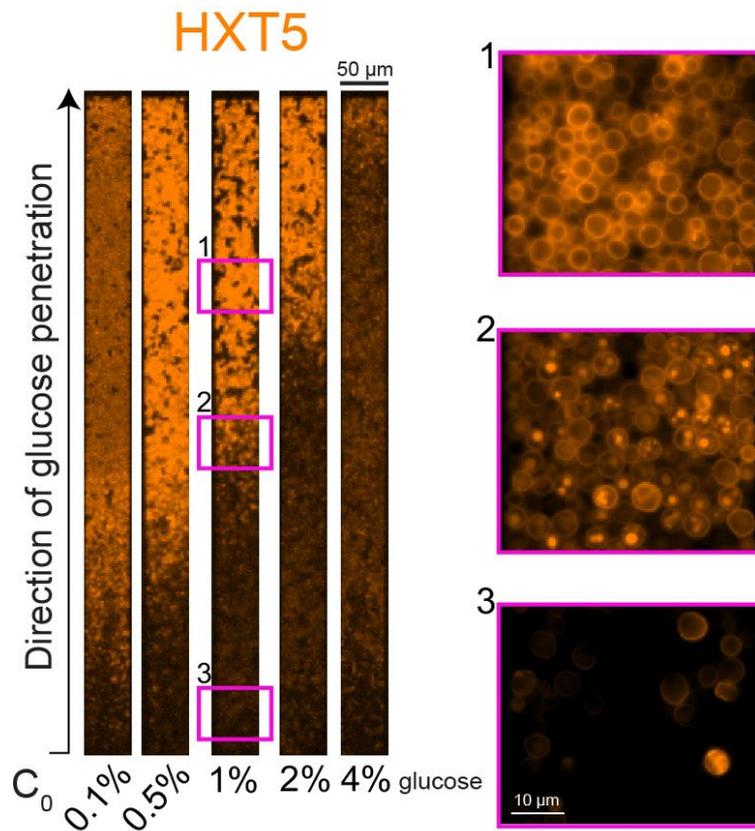


Figure 61 Close up of three different positions inside a colony.  $C_0$  represents the position of the glucose source. Glucose transporter HXT5 is expressed when cell enter in the growth arrest. This can be seen at the back of a colony, where glucose is very low or no glucose is present. Most of the cells have Hxt5p localized on the membrane. Close to the glucose source almost no fluorescence is detected and cells repress the expression of HXT5 in that area. In the transition phase, Hxt5p is targeted for endocytosis and degraded in the vacuole.

## Glucose repression in the structured environment of a yeast colony

Glucose is by far the most preferred carbon source of yeast *S. cerevisiae*. It comes as no surprise that cells developed many strategies to control their metabolism in presence and absence of glucose. When glucose is present, cells want to make the best out of it, so they shut down all the other genes involved in utilization of alternative carbon sources, respiration, and gluconeogenesis. This phenomenon is called glucose repression. One of the most important actors in glucose repression, if not the most important actor along with the Snf1p kinase, is the transcription factor Mig1p which represses the transcription of genes whose expression is shut off when glucose is present. Precisely because of this tight connection with glucose concentrations, we chose to study this gene in more detail.

So far, I quantified the expression of different genes by imaging colonies at low magnification. Doing the same with MIG1 is impossible because we see homogenous levels of expression along the colony in all conditions. The reason for this is that Mig1p localizes in the nucleus when glucose is present so that it can repress other genes, and when glucose is absent it is localized in the cytoplasm. To observe localization in the nucleus we need to image the colony at high magnification.

In low glucose concentrations of 0.1% (55.5 mM) Mig1p is localized in the nucleus only in the parts of the colony that are close to the glucose source, in the other parts of the colony it is present only in the cytoplasm (Figure 62 A). As we increase starting glucose concentrations the parts of the colony that have Mig1p localized in the nucleus increase too, all the way up to the high glucose concentrations of 4% (222 mM) where we see localization of Mig1p in the nucleus along the whole colony except in the part most distant from the glucose source. In that part of the colony, the proportion of cells that have Mig1p localized in the nucleus starts to decline rapidly.

To compare this with cells grown in homogeneous environments we looked at data from the literature. We found two recent publications that used the same strain background as we did. In the Springer lab they grew cells in bulk culture and

then imaged single cells. They counted the fraction of the cells with Mig1p localized to the nucleus and concluded that the localization to the nucleus exhibits a sharp transition between 0.0312% (1.7 mM) and 0.0078% (0.43 mM) glucose [205] (Figure 62 B). As a side note, this is exactly the concentration which corresponds to the peak of glucose transporter HXT7 expression, 0.0156% (0.86 mM). In the Elowitz lab they grew cells in a microfluidic device and imaged the response of Mig1p to different glucose concentrations [206]. Mig1p is actually localized to the nucleus in pulses. As glucose concentration increases, so do the number of pulses and their duration (Figure 62 C). They show that the pulsing frequency and pulse duration quickly decreases in low glucose concentrations similar to those in the Springer lab paper. This pulsing type of regulation also explains why we almost never observe 100% localization to the nucleus in our experiments no matter what the glucose concentration is.

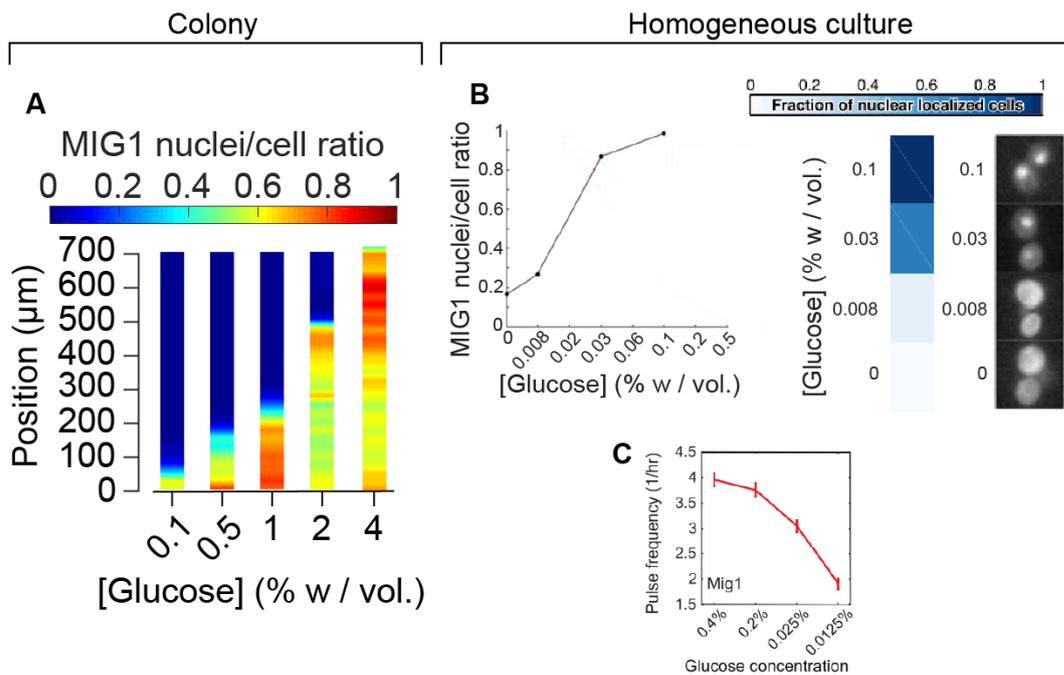


Figure 62 Nuclear localization landscape of the transcription factor MIG1 in different glucose concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, cells that are growing in the presence of glucose localize Mig1p to the nucleus, while cells that are in very low glucose concentrations or in no glucose localize Mig1p in the cytoplasm. (B, C) In homogeneous cultures, Mig1p is localized to the nucleus in very low glucose concentrations and in no glucose. Also, the pulse frequency is higher in higher glucose concentrations. Figures adapted from [205], [206]. Additional figure can be found here (Figure 103).

To quantify the position of localization better, we fitted a sigmoidal curve to our data since the localization ratio follows a sigmoidal pattern, Mig1p is localized

in high fraction in the nucleus or not localized at all, and between these states there is a quick transition. By fitting a sigmoidal curve, we could calculate the position of half maximum nuclear localization, in between the two states (Figure 63). This position should also correspond to the area of low glucose concentration, close to 0.0156% (0.86 mM).

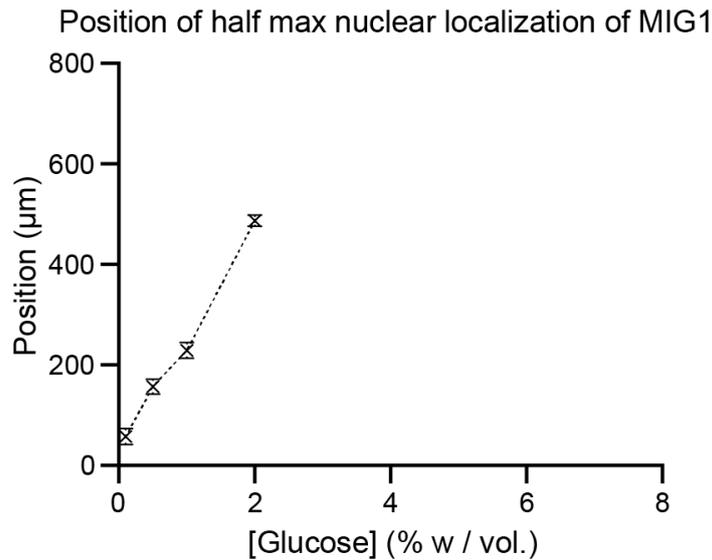


Figure 63 Position of the half maximum nuclear localization of the transcription factor MIG1 in different glucose concentrations. As starting glucose concentration increases, the half maximum nuclear localization moves deeper into the colony.

If we divide the colony in three parts, one close to the nutrient source, second at the transition phase, and third at the back of the colony, we can see differences between single cells (Figure 64). In the parts of the colony where there should be no glucose, Mig1p is localized in the cytoplasm. Close to the glucose source the majority of cells have Mig1p localized to the nucleus. In the transition phase a smaller proportion of the cells have Mig1p localized in the nucleus and we often see a sharp delimitation between the two subpopulations.

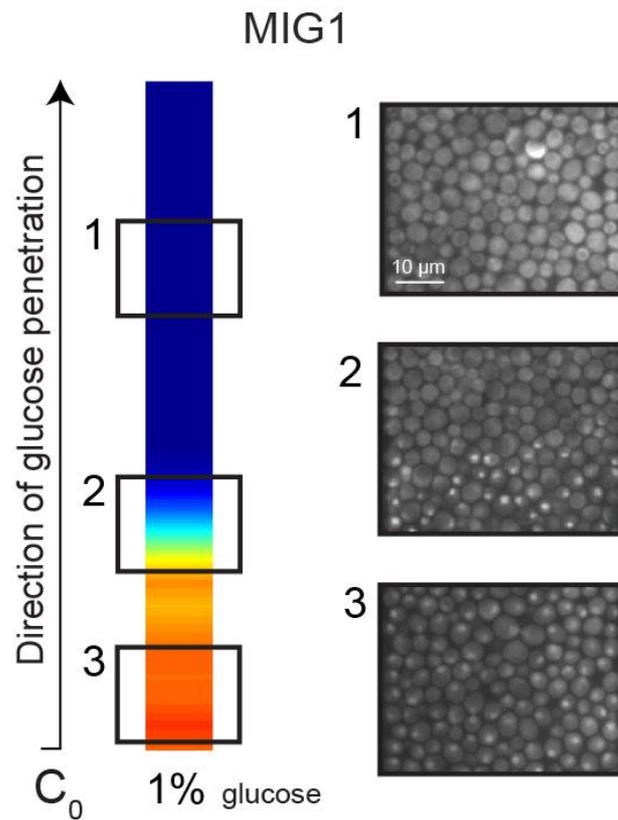


Figure 64 Close up of three different positions inside a colony.  $C_0$  represents the position of the glucose source. Transcription factor Mig1p is localized to the nucleus when glucose is present in the environment. Once the glucose concentration becomes very low or when there is no more glucose, Mig1p is localized to the cytoplasm.

## **Fermentation and respiration in the structured environment of a yeast colony**

Fermentation and respiration are two major modes of metabolism in yeast. Since fermentation and respiration are very sensitive to glucose concentrations and just because of their overall importance, we decided to study them and see if a colony differentiates into subpopulations that show one mode or the other. PDC1 is overexpressed during fermentation and SDH2 is overexpressed during respiration so we used them as a marker that we can track and quantify.

In low glucose concentrations of 0.1% (55.5 mM) SDH2 is not much expressed right at the beginning of the colony close to the nutrient source, but it quickly gets expressed once the glucose gets depleted (Figure 65 A). PDC1, on the other hand, shows almost no overexpression throughout the whole colony except perhaps some indication at the beginning. However, when we increase the starting glucose concentration PDC1 is overexpressed in ever deeper parts of the colony all the way up to the high glucose concentrations of 4% (222 mM) where it dominates throughout the whole colony right until the very end when it starts to decline. In contrast to that, SDH2 overexpression is inhibited ever deeper in the colony as we increase the starting glucose concentration and in 4% (222 mM) glucose it is basically shut down throughout the whole colony. The best example of differentiation into two subpopulations, one that ferments and the other that respire, is seen in 1% (55.5 mM) starting glucose concentration. In almost the first half of the colony closer to the glucose source PDC1 is overexpressed, meaning that this is the part of the colony that ferments, and in the other half of the colony where resources are scarce SDH2 is overexpressed, meaning that this is the part of the colony that respire. It is important to note here that PDMS, the material our microfluidic chip is made of, is porous to gases so we do not expect oxygen gradients to form.

In batch cultures, PDC1 starts to be overexpressed at glucose concentrations around 0.01% (0.55 mM) and then the expression quickly grows up to around 0.1% (5.5 mM) when it starts to level off, but still slowly increasing as glucose concentration is increased. The opposite happens with SDH2. It is repressed in high glucose concentrations and then it starts to be expressed in glucose concentrations

between 0.1% (5.5 mM) and 0.01% (0.55 mM), and also when no glucose is present (Figure 65 B).

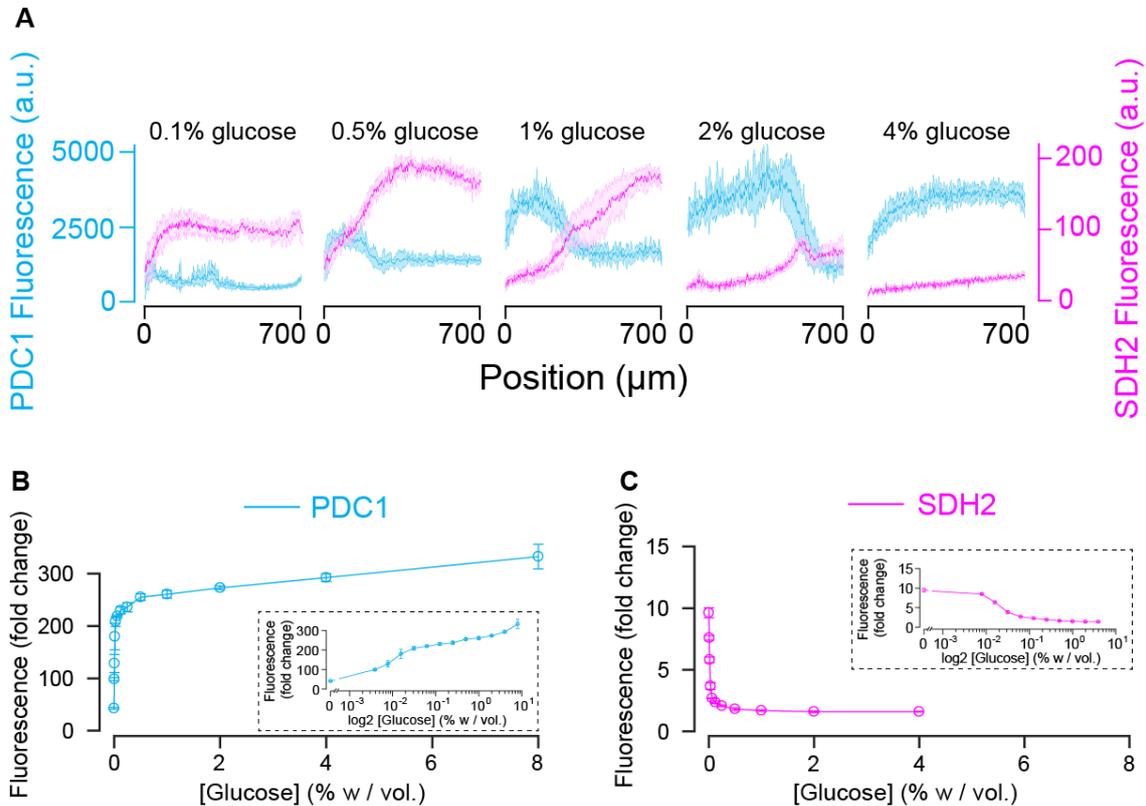


Figure 65 Gene expression landscape of pyruvate decarboxylase PDC1 and succinate dehydrogenase SDH2 in different glucose concentrations. PDC1 is overexpressed during fermentations and SDH2 is overexpressed during respiration. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, two subpopulations differentiate. One that has fermentative metabolism where glucose is abundant and the other that has respiratory metabolism where glucose is scarce. Depending on the starting glucose concentrations, one or the other dominates. (B) In batch cultures, PDC1 is overexpressed in high glucose concentrations and SDH2 is overexpressed in low glucose concentrations or when no glucose is present (main figure: linear x-axis; insert:  $\log_2$  x-axis).

To quantify the position of expression better, we fitted a sigmoidal curve to our data since the expression in most cases follows a sigmoidal pattern. We calculated the position of half maximum expression in between two states where we can approximately say that fermentation and respiration are on or off. This position should also correspond to the area of low glucose concentration, close to 0.01% (0.55 mM). In the concentrations where we could compare the two types of metabolism, the switch for both genes happened at similar positions (Figure 66).

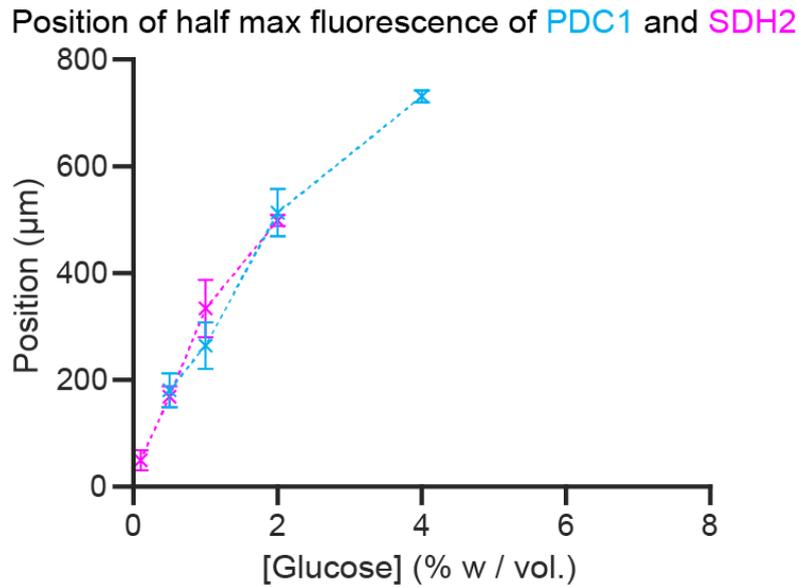


Figure 66 Position of the half maximum fluorescence of pyruvate decarboxylase PDC1 and succinate dehydrogenase SDH2 in different glucose concentrations. As starting glucose concentration increases, the half maximum expression moves deeper into the colony. The position of the half maximum doesn't move linearly with the increase of starting glucose concentration, but it seems to slow down as starting glucose concentration is increased.

The example in 1% (55.5 mM) starting glucose concentration is interesting since colony so clearly differentiates, that we can take a closer look at what is happening in the colony from the point when just a couple of dozens of cells are in the cell chamber to the time when a full colony is formed. When cells that have SDH2 endogenously tagged with GFP are loaded in the cell chamber they express almost no fluorescence (Figure 67 A). But at the point when glucose concentration becomes sufficiently low, cells start to express SDH2 and pattern of expression appears. As colony grows, SDH2 pattern gets larger depending on the size of the colony, until the colony reaches its definitive size in the cell chamber and the pattern takes a more constant form. In contrast to that, PDC1 is already expressed when loaded on the device (due to the preculturing conditions, but also because it has a relatively high basal expression). As colony grows it is hard to distinguish the front and the back, but once a full colony is established we can see an overexpression of PDC1 in the areas close to the glucose source (Figure 67 B).

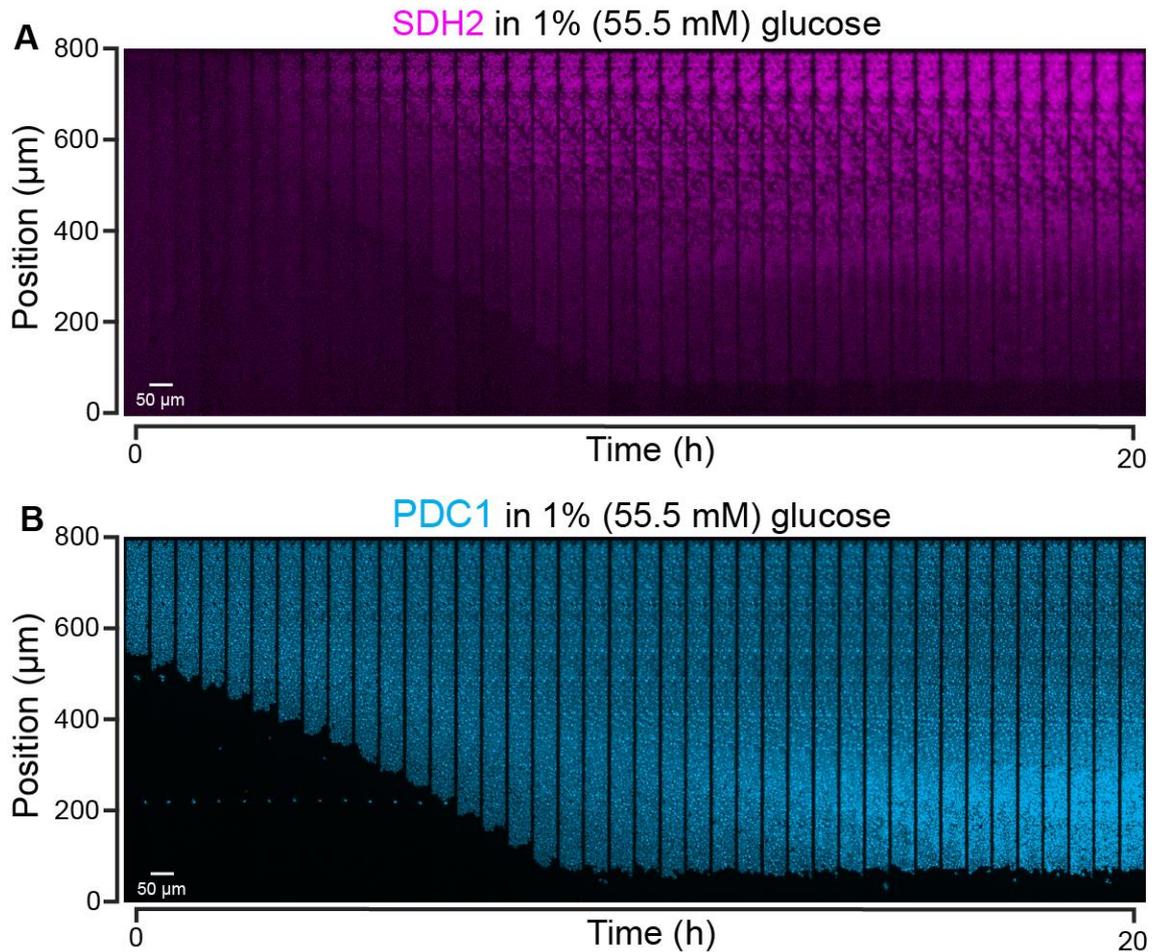


Figure 67 Kymograph of pyruvate decarboxylase PDC1 and succinate dehydrogenase SDH2 in 1% (55.5 mM) starting glucose concentration. Time step between two images of the same colony 30 minutes. Position 0  $\mu\text{m}$  in this case reflects the beginning of the cell chamber and not the starting glucose concentration as one might observe that the edge of the colony is not at the same position as the cell chamber to nutrient channel junction. Time 0 h is time when the cells were loaded and the number of loaded cells is not always constant. (A) As colony with SDH2 endogenously fused with GFP is growing, at one moment SDH2 starts to be expressed at the back of the colony where glucose is scarce. The bigger the colony grows, the bigger the area of SDH2 expression gets. (B) PDC1 is overexpressed in high glucose conditions. It seems like PDC1 overexpression takes some time to be fully visible. But once it is turned on, we can clearly see overexpression in the area close to the glucose source.

When we image the colony at high magnification we see differences in expression more precisely. If we divide the colony in two parts, one close to the nutrient source where fermentation is supposed to occur and the other at the back of the colony where respiration should be turned on, we can see differences between single cells (Figure 68). In the parts of the colony where glucose is abundant PDC1 is mostly localized in the cytoplasm and SDH2 is barely expressed in mitochondria. It has been previously shown that addition of glucose to a glucose-limited culture causes the SDH2 mRNA level to fall rapidly [207]. In contrast to glucose rich parts of

the colony, areas of the colony where glucose is scarce or where there is no glucose at all show high expression of SDH2 in mitochondria and low expression of PDC1 in one part of the cells and some kind of focalization in the other part. In a recent article O'Shea and Zid studied PDC1 and concluded that upon glucose starvation PDC1 mRNAs localize to so called P bodies as foci in cytoplasm [208].

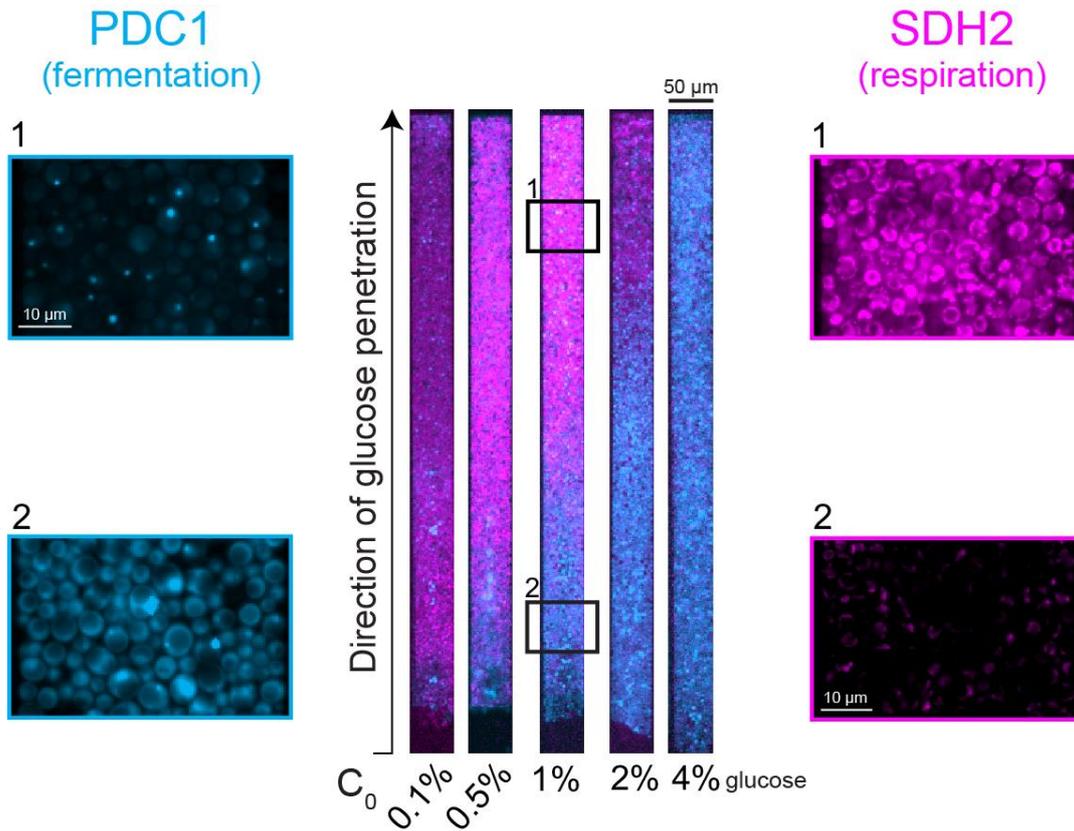


Figure 68 Close up of two different positions inside a colony.  $C_0$  represents the position of the glucose source. Pyruvate decarboxylase PDC1 is overexpressed in high glucose concentrations and succinate dehydrogenase SDH2 is overexpressed in low glucose concentrations or when there is no glucose present.

## **Glycolysis in the structured environment of a yeast colony**

Finally, we looked at the first irreversible step of glycolysis. Three genes catalyse the reaction of glucose phosphorylation: hexokinase 1 (HXK1), hexokinase 2 (HXK2), and glucokinase 1 (GLK1). GLK1 expression was too low for detection in colonies so we could not quantify it. In batch cultures, HXK1 shows a peak-like gene expression reminiscent of glucose transporter HXT7 expression pattern (Figure 69 B). The expression reaches a maximum at 0.0156% (0.86 mM) and it seems to be repressed at high glucose concentrations as well as when no glucose is present. HXK2 on the other hand is constantly expressed between 0.1% (5.5 mM) and 8% (444 mM) glucose and there seems to be a slight peak of expression at 0.0078% (0.43 mM) glucose before it falls down significantly when there is no glucose, but it is not completely shut off.

When we imaged their expression in a colony, we indeed noticed peaks of expression (Figure 69 A). They appeared in 1% (55.5 mM) and 2% (111 mM) starting glucose conditions. When we increased the glucose concentration to 8% (444 mM), HXK1 was expressed very low and HXK2 was expressed more than HXK1 as expected from the batch culture experiments. We quantified the position of peaks in glucose concentrations where it was possible and they appeared in the similar area of low glucose concentrations. In addition to that, HXK2 peak of expression might appear to be slowing down as glucose is increased to very high concentrations (Figure 70).

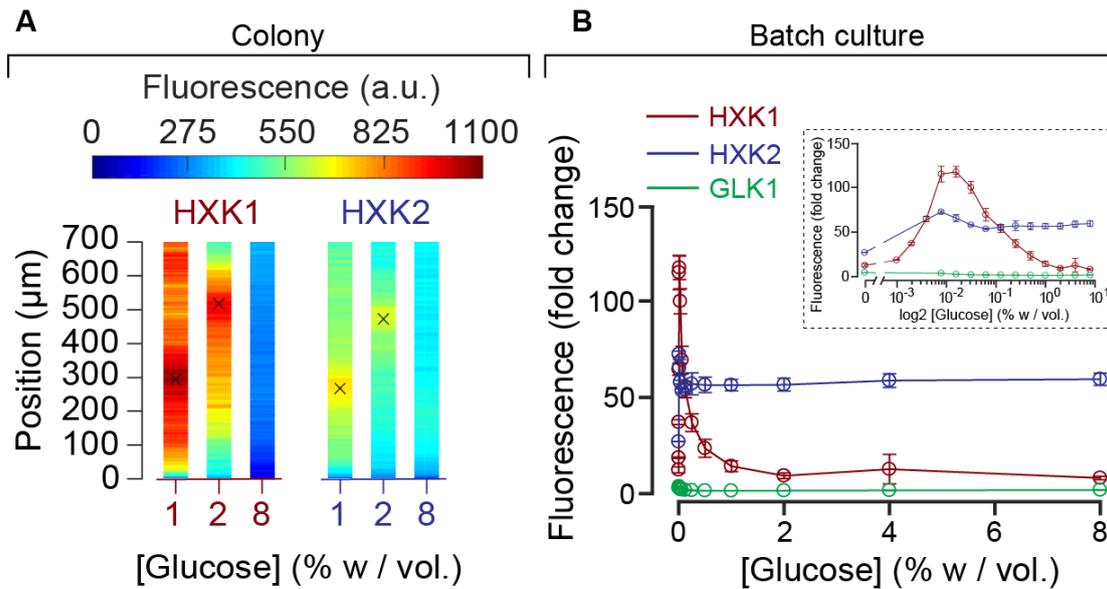


Figure 69 Gene expression landscape of hexokinase 1 (HXK1), hexokinase 2 (HXK2), and glucokinase 1 (GLK1) in different glucose concentrations. GLK1 expression was too low for detection in a colony. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) In colonies, both HXK1 and HXK2 show peaks of expression in starting glucose concentrations of 1% (55.5 mM) and 2% (111 mM). In 8% (444 mM) glucose, HXK1 is expressed at low levels and HXK2 shows higher expression levels. Black x represents the peak of expression. Fluorescence values represent measured values after the background is removed. (B) In batch cultures, GLK1 is expressed at almost too low levels to detect. HXK1 shows a peak like expression with a maximum in low glucose concentration. HXK2 is expressed at constant levels in high glucose concentrations and it shows a slight peak of expression in 0.0078% (0.43 mM) glucose (main figure: linear x-axis; insert:  $\log_2$  x-axis). Additional figures can be found here (Figure 104, Figure 105).

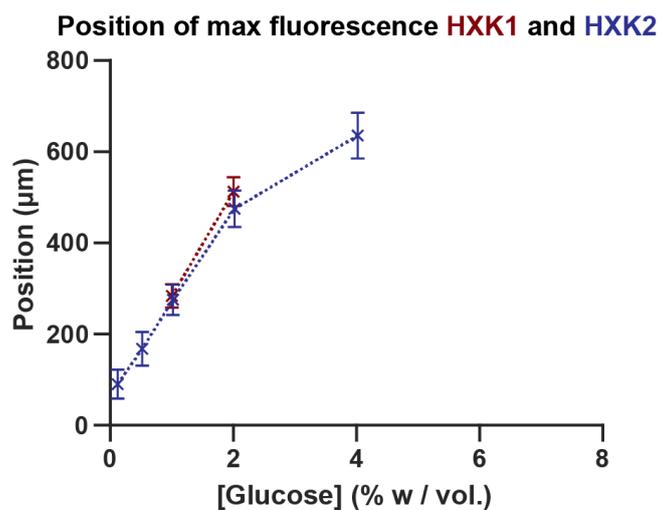


Figure 70 Peaks of expression of hexokinase 1 (HXK1) and hexokinase 2 (HXK2). As starting glucose concentration increases, the peak of maximum expression moves deeper into the colony since cells are adapting to the environment inside the colony.

We can get the clearest picture about HXK1 and HXK2 if we take a closer look at what is happening in the colony from the point when just a couple of dozens of cells are in the cell chamber to the time when a full colony is formed. As the colony grows, cells start to adapt to their environment and turn on the expression of their endogenously GFP-tagged genes HXK1 or HXK2. We can see that HXK1 turns on when glucose becomes sufficiently low within the colony and once the whole colony is established a clear peak of expression starts to appear. In 1% (55.5 mM) glucose it is closer to the glucose source and in 2% (111 mM) it is further away from the glucose source (Figure 71 A, C). We can also see that at the back of a colony patterns of previous expression are conserved. When glucose is depleted HXK1 forms similar foci in cytoplasm as PDC1 when it localizes to P bodies [208]. HXK2 is a bit more complex, it was one of the first metabolic enzymes described as a multifunctional protein [209]. HXK2 has two roles and two localizations, in the cytoplasm it catalyzes glycolysis and in the nucleus, it is a regulator of gene transcription of several MIG1-regulated genes. Nevertheless, we can see a peak of expression in low glucose as we might predict from the batch culture. In 1% (55.5 mM) glucose it appears closer to the glucose source and in 2% (111 mM) glucose it appears further away (Figure 71 B, D).

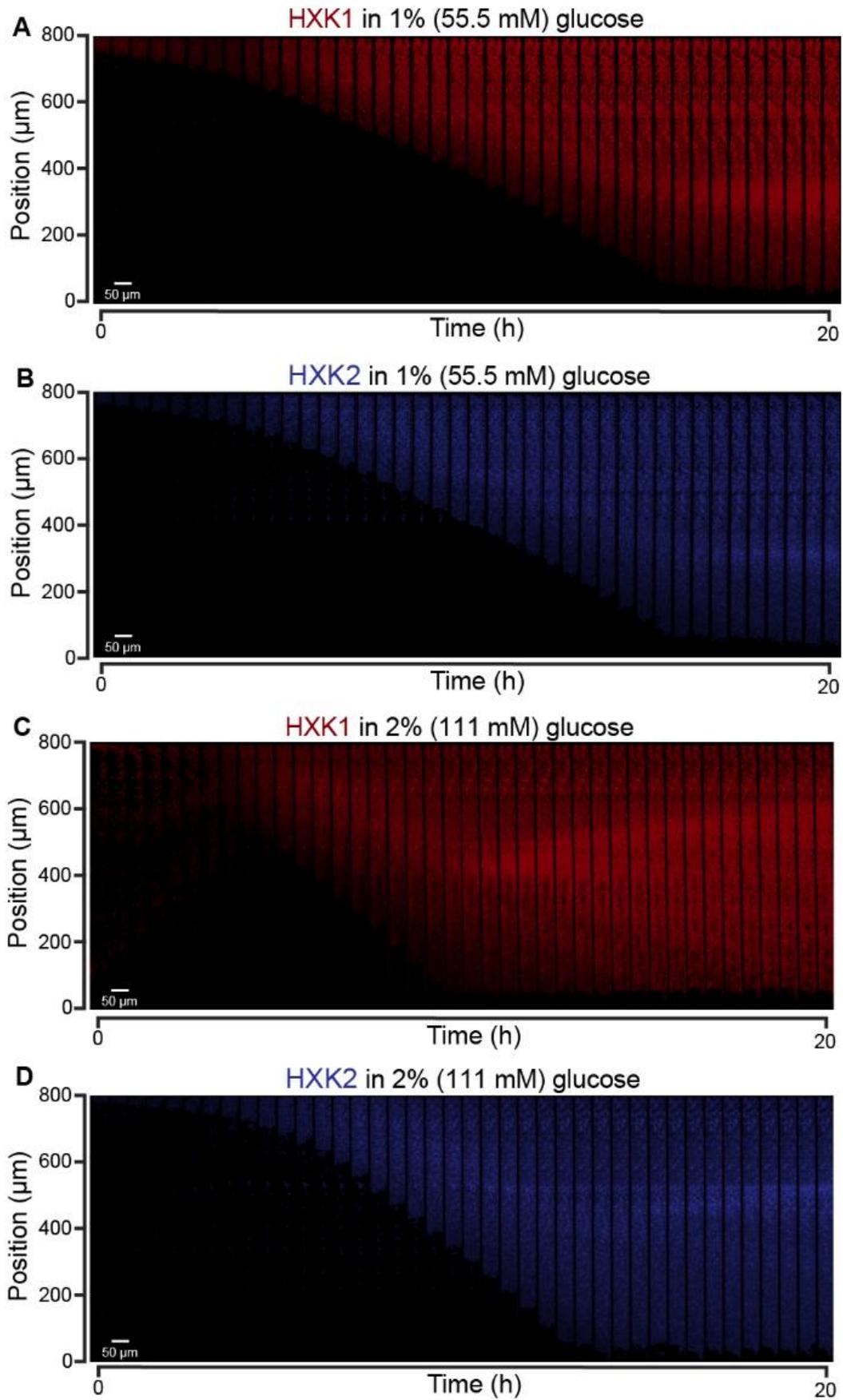


Figure 71 (previous page) Kymograph of hexokinase 1 (HXK1) and hexokinase 2 (HXK2) in 1% (55.5 mM) and 2% (111 mM) starting glucose concentration. Time step between two images of the same colony is 30 minutes. Position 0  $\mu\text{m}$  in this case reflects the beginning of the cell chamber and not the starting glucose concentration as one might observe that the edge of the colony is not at the same position as the cell chamber to nutrient channel junction. Time 0 h is time when the cells were loaded and the number of loaded cells is not always constant. As GFP-labelled HXK1 and HXK2 colony is expanding, a peak-like pattern of expression starts to appear in the area of low glucose concentrations. In 1% glucose the peak is closer to the glucose source and in 2% glucose it is further away.

## **Landscape of gene expression of other genes and transcription factor activity confirms the inferred glucose gradient**

To summarize what has been shown so far related to gene expression within colonies I present two figures that combine the most interesting genes and position of their expression represented as pictures of real colonies (Figure 72) and a combination of different quantification methods mentioned earlier, position of peaks of maximum expression and position of half maximum expression where both should correspond to the area of low glucose concentration around 0.01% (0.55 mM) (Figure 73). The expression levels of HXT7 seem to be enough to define the distance at which the glucose level has significantly decreased to reach 0.0156% (0.86 mM) concentration. At such low concentration, glucose becomes so scarce that it changes the cell metabolism. We hypothesized that we should thus be able to see a change of expression and/or localization of other key genes involved in glucose metabolism. To start with, we looked at cells at higher magnification (60x) to check the localization of gene expression of HXTs. As expected, glucose transporters were localized at the membrane in areas where the levels of expression were the highest, indicating that they actively play a role of glucose transport in these areas. In contrast, deeper in the colony, we still observed (lower) fluorescence due to the long lifetime of GFP fused proteins and almost no dilution through division, but the fluorescence was localized in vacuoles, indicating that the transporters were targeted by the cells for degradation. Glucose transporter HXT5 was particularly useful to study since it is expressed when cells enter into growth arrest when glucose is very low or when there is no glucose at all. We then mapped other genes specific for different parts of glucose metabolism. We looked at localization of the gene MIG1, an important regulator of the glucose metabolism, which is located in

the nucleus in presence of glucose. Observing cells at high magnification we quantified the distance at which MIG1 transitions from nuclear to cytoplasmic localization. This distance is in good agreement with the one obtained by HXT7 profiling. Similarly, we looked at the expression of PDC1 and SDH2, two genes that are overexpressed during fermentation or respiration and can be used as markers for these two distinctive modes of metabolism. Maps of expression were anti-correlated, indicating that there is indeed a structure of at least two subpopulations inside the colony, with a smooth transition from a fermentative metabolism at the front close to higher glucose concentrations and a respiratory metabolism at the back where glucose is scarce. The transition occurs at a distance which is close to the change of MIG1 localization and the peak of expression of HXT7. We also looked at the first irreversible step of glycolysis which is catalysed by three genes: HXK1, HXK2, and GLK1. While levels of GLK1 were too low to be detected, both HXK1 and HXK2 showed a peak like expression within a colony in the areas of low glucose which in most cases correlated well with the expression pattern of other genes.

**Taken together, we have demonstrated that an assembly of cells is not a collection of identical cells, but on the contrary that they collectively interact through long range interactions, self-inflicting a glucose gradient and more generally a microenvironment that can be monitored by observing landscapes of gene expression.**

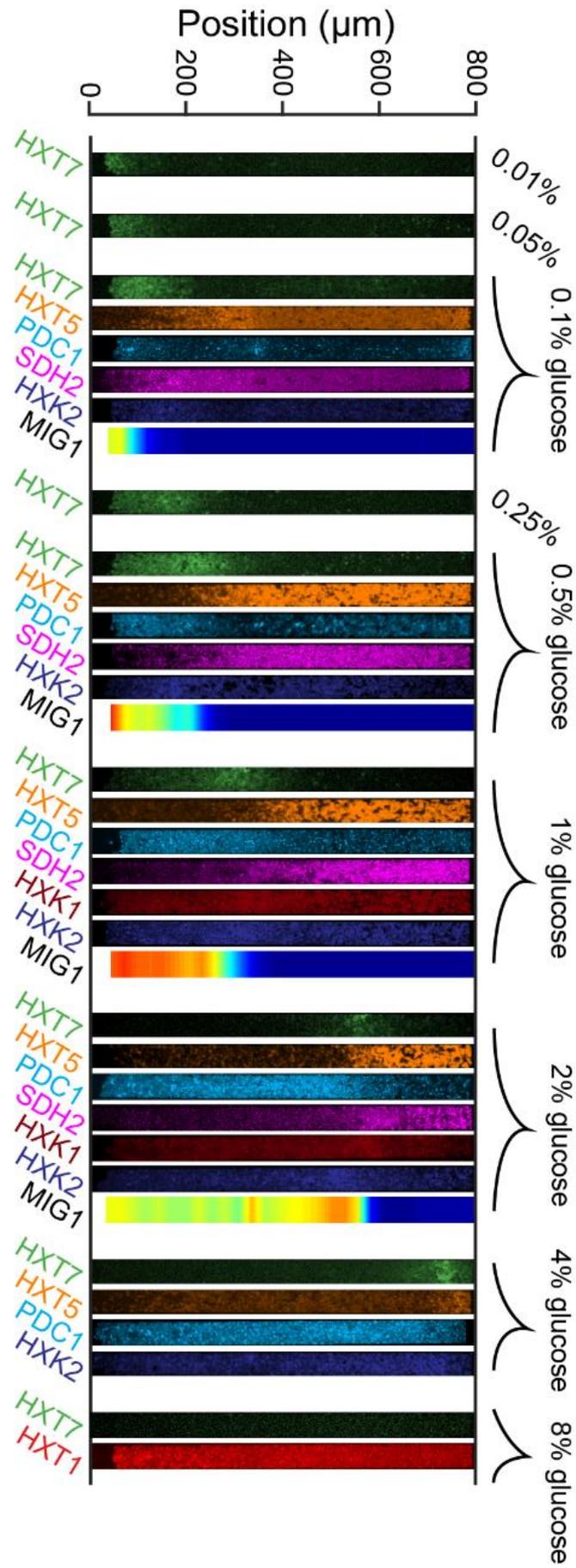


Figure 72 (previous page) Expression patterns of genes related to the glucose metabolism in different glucose concentrations. All genes except MIG1 (because localization cannot be seen at low magnifications) represent pictures of real colonies. Position 0  $\mu\text{m}$  in this case reflects the beginning of the cell chamber and not the starting glucose concentration as one might observe that the edge of the colony is not at the same position as the cell chamber to nutrient channel junction (position of MIG1 panel is adjusted since measurements are adjusted to represent position 0  $\mu\text{m}$  as position of the glucose source).

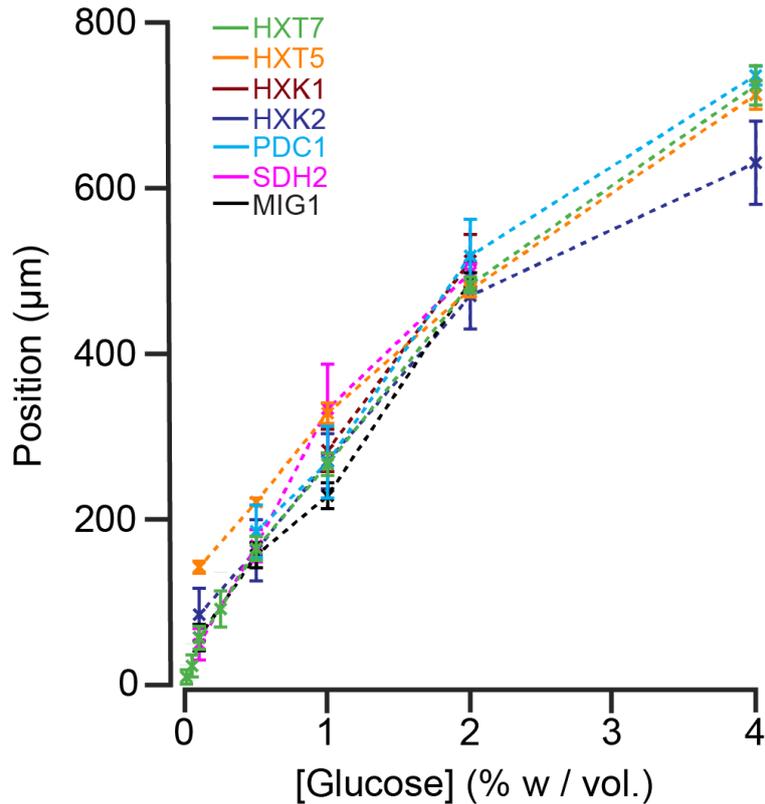


Figure 73 Positions of peaks of maximum expression or half maximum expression of different genes as a function of starting glucose concentration. Data points of HXT7, HXK1, and HXK2 represent the peak of expression. Data points of HXT5, PDC1, SDH2, and MIG1 represent the position of half maximum expression. All data points should correspond to the area of low glucose concentrations in the vicinity of 0.01% (0.55 mM). In most of the cases all genes converge to the similar position of expression and the trend doesn't seem to be linear with the increase of starting glucose concentration, but rather the progress into the colony seems to slow down as we increase the starting glucose concentration.

## Conclusion

When single cells grow together they form a complex collective behaviour derived from simple rules, like nutrient uptake dynamics. I presented here a microfluidic device which is possible to mimic growth dynamics closer to the ones happening in nature. Its comparative advantages to other common methods used when looking at growth and differentiation of colonies are several:

- I. Only a monolayer of yeast cells can be grown inside the cell chamber which leads to the direct access to single cell behaviour within colonies
- II. Dimensions and architecture of nutrient channels and cell chambers can be changed at experimenters will, leading to a control over spatial structure of the cell assembly
- III. The dynamics of growth and gene expression can be constantly monitored and imaged with time-lapse microscopy
- IV. Environments can be easily maintained, changed, and controlled

Through long optimization, trials, fails, and successes, we managed to build a device, “The Yeast Machine”, that can sustain growth of a monolayer of yeast colony for almost one week. Its dimensions are long enough that we can observe populations that are growing at different rates and that are not growing at all. We demonstrated that we can look at the global colony expansion as well as local growth within colony. We also demonstrated that we can observe colony at global scale with low magnification and at local scale with high magnification.

The expansion rate of a colony increases with time, as more and more cells divide and have access to glucose. At one point glucose is depleted and the number of cells that are growing is fixed which also saturates the expansion. When we change the starting glucose concentration, the expansion of the colony changes too. As starting glucose concentrations is increased, the expansion velocity increases with it, but not linearly, probably due to the uptake kinetics. The expansion velocity as a function of glucose concentration eventually levels off due to the dimensions of the cell chamber, because at one point  $\sim 4\%$  (222 mM) glucose concentration most of the cells within a colony grow, and the addition of glucose does not significantly change the growth dynamics. This is to large extant expected as we know from the

homogeneous liquid culture experiments that growth rate in yeast saturates very quickly, already at concentration lower than 0.05% (2.75 mM) glucose.

Once the whole colony is formed within the cell chamber, the local growth can be observed and measured as local velocity within a colony. We thus see a landscape of growth dynamics, where not all cells grow at the same velocity (which would imply an exponential growth). As glucose concentration is increased, longer parts of colony grow and the local velocity increases. We can distinguish parts of the colony that are growing and parts that are not growing, which gives us an estimate of the position of glucose depletion.

We can also monitor the landscape of gene expression which emerges naturally from the function of each single cell in the colony. This is mainly due to the formation of glucose concentration gradient, which is set by the interplay of glucose diffusion and glucose uptake by the cells themselves. We can say that cells “self-inflict” this gradient on themselves and form a “long-range metabolic interaction” within a colony. The interaction between cells and the environment goes both ways. Cells phenotype is set by the environment, but also cells construct the environment through the uptake of nutrients and the release of molecules into the environment. We used seven major glucose transporter genes HXT1-7, which are expressed in different glucose concentrations, to observe their differentiation within a colony and to quantify the glucose concentration in the environment. To this end, we combined the experiments with colonies with the experiments in liquid batch cultures. For glucose gradient reconstruction, we used glucose transporter genes HXT1 and HXT7, which have an opposite expression pattern in glucose, one is expressed in high and the other in low glucose concentrations. Based on their expression pattern we managed to reconstruct the glucose concentration gradient, which showed a faster depletion of glucose in higher glucose concentration and the slowing down of glucose uptake in lower glucose concentrations. Interestingly, the pattern of all tested glucose transporters could be predicted from the expression patterns in bulk cultures, in a way that we can guess in which parts of the colony will the pattern appear and how it will relate to other glucose transporters.

With this clear phenotypic differentiation in mind, we embarked upon the study of other genes and phenomena related to glucose concentration. We wanted to see if we can observe the emergence of different subpopulations within a colony and will it follow the glucose concentration gradient that we established in the experiments with glucose transporters. We first measured the growth arrest with the help of glucose transporter HXT5 which is expressed during growth arrest and

indeed we saw a clear differentiation between growing and non-growing population, which also depended on the starting glucose concentration. As glucose concentration was increased, ever larger parts of the colony grew and had HXT5 expression repressed. These observations were also in line with the local velocity measurements, where we also observed separation between growing and non-growing subpopulation and their dependence on the glucose concentration.

Since glucose is the preferred carbon source of yeast *Saccharomyces cerevisiae*, a whole glucose repression mechanism has evolved around the need of repressing genes for the utilization of other carbon sources. Major transcription factor that mediates this repression is MIG1, which localizes to nucleus when glucose is present to repress other genes, and it localizes to cytoplasm when glucose is not present. Looking cells with the high magnification we quantified the localization of MIG1 and again observed the emergence of populations that have MIG1 localized in the nucleus or in the cytoplasm. Yet again, this pattern followed the starting glucose concentrations. As glucose was increased, glucose repression reached further within a colony.

Fermentation and respiration are two major modes of glucose metabolism in yeast. We used genes that are specifically overexpressed in one or the other mode. Again, we observed a clear differentiation between two subpopulations, one that is fermenting when glucose is high and the other that is respiring when glucose is low. Their relative size also depended on starting glucose concentrations, as larger parts of the colony fermented as glucose was increased.

Quantification of the genes involved in the first step of glycolysis, hexokinase 1 (HXK1) and hexokinase 2 (HXK2) also showed a good agreement with the expected pattern of expression which depends on the local glucose concentration.

At the end, we combined all the genes together and quantified positions where we could expect glucose to be low, both from batch and colony experiments. This included peaks of gene expression like in HXT7, transitions between fermentation and respiration like in PDC1 and SDH2, transition to growth arrest in HXT5, transition to localization to cytoplasm by MIG1, and peaks of expression of HXK1 and HXK2. Positions of peaks and transitions showed a good agreement with the expected areas of low glucose concentrations, reassuring our argument that we see the formation of glucose concentration gradient and that cells differentiate and self-organize into complex entities when grown together.

We think that this method is an excellent model for growth and phenotypic differentiation of microorganisms in the wild and that it can be used to study different phenomena that stem from self-organization of single cells into colonies. We hope that these are humble beginnings of a method that could yield with interesting discoveries in different fields like aging, cooperation-competition and experimental evolution, chemical ecology, multi-species interaction, and microbiome engineering.

However, we did not stop only at this point. We were also interested to see what will happen if we limit some other nutrient, like amino acids. Not only that, we wanted to see what kind of interaction arises from the interplay of glucose and amino acids limitations. In addition to that, we wanted to strip off the possibility of cells to uptake and sense glucose, or better to say, to see what will happen if cells depend only on a single glucose transporter and not on a more than a dozen of them. I tackle these topics further in the text.

## Additional research

### Growth and gene expression landscape in amino acid limitation in yeast colonies

As we demonstrated in our work, glucose does become limiting for growth inside colonies. But if glucose becomes limiting through the interplay of diffusion and uptake, surely all other nutrients form gradients and microenvironments inside colonies as well. To check this, we decided to use an inherent property of our strains (and most of the laboratory yeast strains, as a matter of fact): auxotrophy. To facilitate genetics and genomics experiments researchers have been using auxotrophic markers for decades [210]–[213]. They would do a mutation in a gene that encodes a key enzyme in the biosynthetic pathway of a certain amino acid and use it for selection, making cells that carry such mutation dependent on supplementation of said amino acid in media. It has been thought for a very long time that as long as an amino acid that yeast cannot produce is supplemented in the media, there will be little or no influence on an experiment. However, a recent paper by Ralser's group reported a wide range of consequences of different markers across different strains on cell's metabolome, even in absence of noticeable growth effects [214]–[216]. This just shows how important it is to characterize well both cell's internal environment as well as cell's external environment since cell's genotype to phenotype relation will depend on integration of all these complex signals into a dynamic response. A good starting point on interstrain variability of yeast *S. cerevisiae* in response to environmental factors can be found in a recent paper from Springer's group [217]. They looked at differences in yeast galactose pathway response across many strains to an environment with glucose and galactose concentrations in different ratios. This trend of realizing the importance of internal and external environments, especially in conjunction with emerging properties of cell assemblies (either isogenic cells that I am studying or multispecies assemblies), is becoming more evident from recently published papers but also from a variety of ongoing and nascent projects.

Although our microfluidic system could be used to probe all the ranges of nutrients that diffuse and are consumed by cells, leading to a subsequent differentiation according to specific microenvironment, the reason we used auxotrophy is somewhat simpler. Our hypothesis was that colony will differentiate into two subpopulations, one that is growing and the other that is not, once the essential amino acids are depleted from the colony. Since we mixed amino acid concentrations with different glucose concentrations that we feed our colonies with, the consequential response of a colony should depend on the interplay between amino acid and glucose concentrations. To put it simply, one or the other will become a limiting nutrient at a certain position in the colony which could be detected by looking at the growth and even gene expression (Figure 74).

To do this, we decreased the concentration of amino acids five times compared to the previously shown experiments where only glucose concentration is supposed to be limiting. This means that we used 1x concentration of complete supplement mixture (CSM), a well-defined mixture of 14 amino acids among which are four that our background strain S288c BY4741 is lacking, histidine, leucine, methionine, and uracil. Two things are interesting to note here. First one is that scientists commonly use 1x CSM in experiments in well mixed liquid cultures as a part of the well-defined synthetic complete (SC) media, hence the media that we are using to look at potential amino acid limitation in colonies is a well-established complete media in liquid cultures. Second thing to note is that yeast prefer to uptake amino acids from their environment even if they can biosynthesise them by themselves [218]–[220]. This is probably so due to the fact that it “costs” less to uptake amino acids from the environment (many amino acid transporters are not specific or they are specific for a group of amino acids), than what it “costs” to produce new amino acids through a whole biosynthetic pathway.

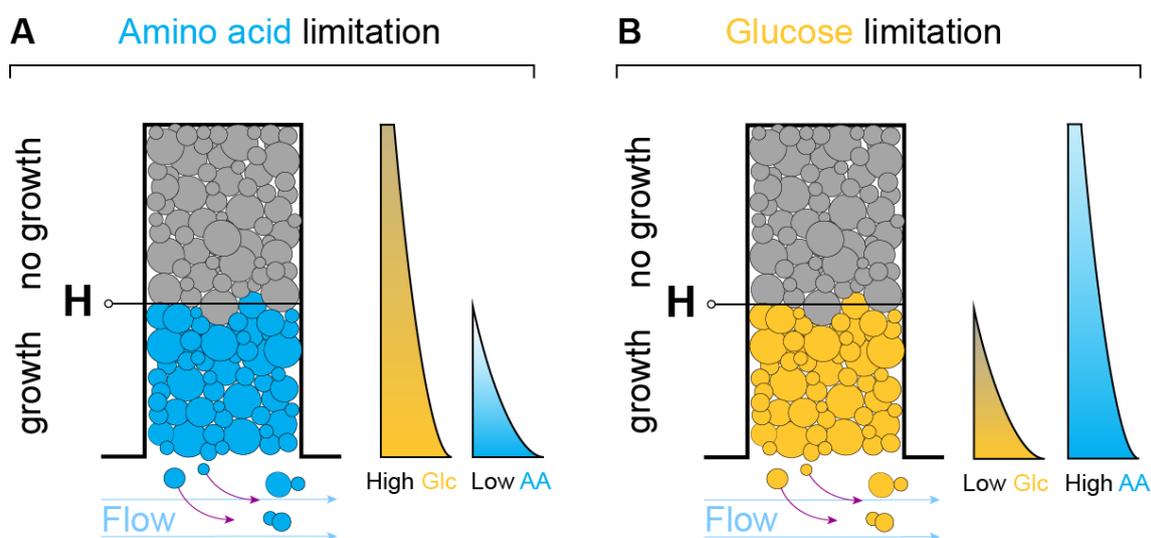


Figure 74 Interplay between limitation of two different nutrients that impact growth inside a colony. (A) If the starting glucose concentration is high enough so that all colony has access to glucose and the starting amino acid concentration is low enough that it will be depleted at some distance (H) within the colony, colony growth will depend on amino acid limitation. (B) If the starting amino acid concentration is high enough so that all colony has access to amino acids and the starting glucose concentration is low enough that it will be depleted at some distance (H) within the colony, colony growth will depend on glucose limitation.

In addition to the auxotrophic strain S288c BY4741 that we used due to its genetic tractability, good characterization, wide use, and access to gene tag and gene deletion libraries, we did try to use a prototrophic strain in our experiments in order to see if we can decrease a level of complexity by eliminating supplementation of amino acids in media. The Murray lab kindly provided us a haploid prototrophic strain yJHK112 based on W303 background [37] but it was interesting to see how differently it behaved when grown in our microfluidic device. At one point in the experiment, around 20 hours after the start, cells in the middle of the colony systematically started to get unusually big, reaching up to 20  $\mu\text{m}$  in diameter. We still do not know what caused this, it has probably something to do with the microenvironment and nutrient gradients formation and/or stress response, but we decided that it would not be worth of pursuit to accommodate this problem since very big cells were clogging the “Yeast Machine” and changing the architecture to deal with big cells would necessarily lead to new rounds of optimization of the whole system which would not fit the scope of this thesis. I hope that one day someone will decide to tackle the reasons why these cells were getting so big as well as characterizing growth and gene expression of prototrophic strains.

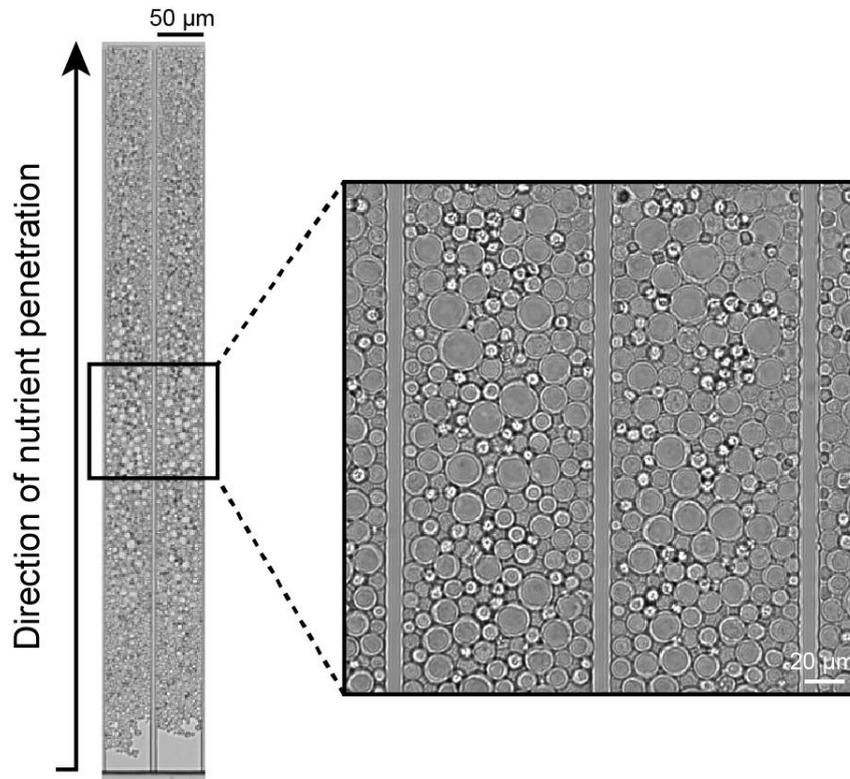


Figure 75 Prototrophic strain based on W303 background after 20 hours of growth. Cells in the middle of the colony started to inflate and get unusually big during growth in the “Yeast Machine”.

We applied the same methods, described before, to quantify growth and gene expression of yeast in, what we call, low amino acid conditions (1x CSM). Under assumption that cells deplete amino acids within a colony and that from the position of depletion they are unable to grow no matter how much glucose we add, we would expect to see a saturation of front velocity as a function of glucose concentration. The reason for this being that no matter how much more glucose we add, a fixed number of layers of cells will grow which is determined by amino acid depletion. For example, if in low amino acid conditions only first 300 μm of colony has access to amino acids, any glucose that is not depleted in the first 300 μm of the colony will not have any effect on the growth anymore. This also means that in the first 300 μm of a colony in low amino acid conditions, glucose will be limiting to growth if it gets depleted up to that position. This is, to some extent, a simplified view of the system as we know that both glucose and amino acids will form gradients within a colony, which will also be coupled with specific growth and uptake rates in different nutrient concentrations.

When we measure the front velocity in low amino acids, we see that the velocity is fixed at 2% (111 mM) glucose and it doesn't change anymore as we

increase starting glucose concentrations (Figure 76). If we compare front velocities in low amino acids and high amino acids, we see a difference in velocities in high glucose concentrations due to the fact that cells are not limited by amino acids and therefore all cells in the colony have sufficient nutrients to grow provided that there is glucose in their microenvironment. A note needs to be given here, as I mentioned earlier in the text, the levelling off of the front velocity in high amino acids and high glucose concentrations is caused by the limiting length of our microfluidic device where at 4% (222 mM) glucose almost all cells have access to it. On the other hand, if we compare front velocities in low amino acids and high amino acids in low starting glucose concentrations, we see an overlap in velocities because in that part glucose is the only limiting nutrient. Somewhere in the area of 0.5% (27.5 mM) and 1% (55.5 mM) glucose, we see a transition between the overlap and the separation of front velocities. It is reasonable to predict that the position where glucose gets depleted if starting glucose concentrations are 0.5% - 1% is close to the position where amino acids get depleted when we feed the colony with low amino acid concentration (1x CSM).

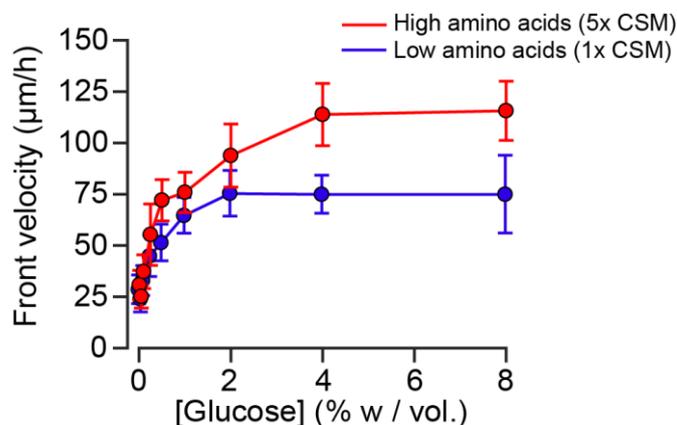


Figure 76 Colony front velocity as a function of starting glucose concentration in high (red) and low (blue) amino acid concentration. Front velocity increases as more glucose is added because cells grow faster and more layers of cells contribute to expansion of the colony, but in low amino acids a plateau is reached and velocities are lower due to the fact that amino acids become depleted and the number of cells that are growing becomes limited.

We also tracked the front position as colony was growing from the inoculation in the cell chamber to the point where it filled out the whole chamber. If

we compare low and high amino acid concentrations with different starting glucose concentrations we again see the separation of velocities in high glucose concentrations and transition to complete overlap in low glucose concentrations (Figure 77).

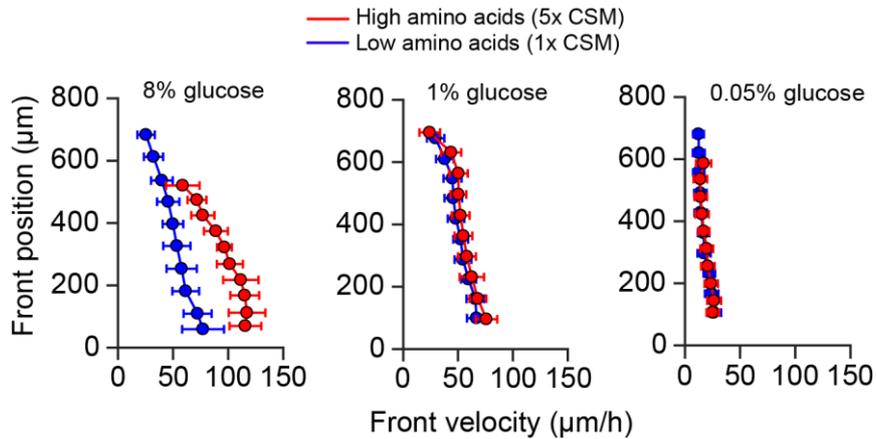


Figure 77 Evolution of colony front velocities as colony grows. Each panel represents colony front velocity at each position measured while colonies were growing in the “Yeast Machine” in different glucose and amino acid concentrations. Depending on the starting glucose and amino acid concentrations, but also diffusion and uptake rates which evolve as a function of local nutrient concentration, the number of cell layers that grow and their growth rates are set.

To better understand low amino acid limitation, we quantified local velocities within a colony once the cells have filled out the whole cell chamber (Figure 78). We used the same method as described before in the case of high starting amino acid concentration (5x CSM). Across all glucose concentrations cells in the back of the colony, in parts that are deprived of amino acids due to their diffusion and uptake by the cells, are not growing. We can, however, notice an increase in local velocities in the area of 200 μm to 300 μm, meaning that at this position amino acids become limiting when glucose is provided in high quantities.

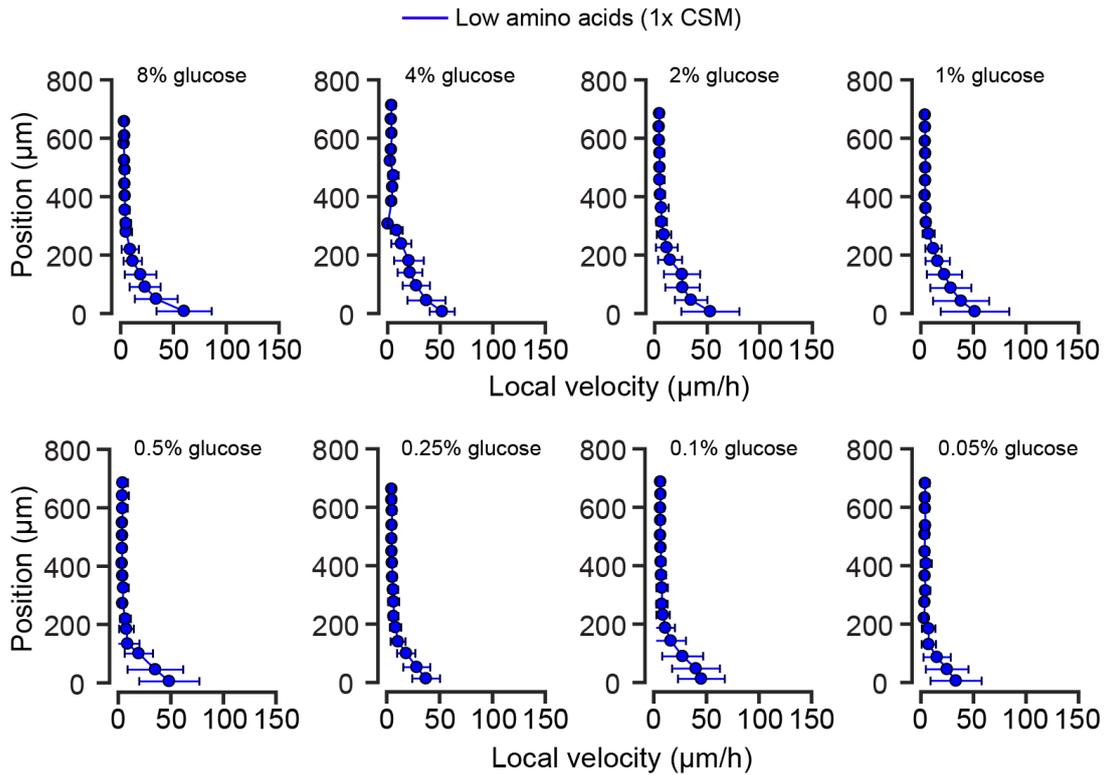


Figure 78 Local velocities within a colony in low starting amino acid concentrations (1x CSM). Each panel represents local velocity within a colony at each position measured while colonies were growing in the “Yeast Machine” across a range of glucose concentrations. Depending on the starting glucose and amino acid concentrations, as well as diffusion and uptake rates, the number of cell layers that grow and their growth rates are set. Position 0  $\mu\text{m}$  is the position of the glucose source.

Again, if we compare the local velocities in high and low amino acid concentrations across a wide range of concentrations, we can see a separation of velocities in very high glucose concentrations where virtually all cells within a colony grow if we provide them with high amino acid concentration, in contrast to low amino acid concentration where cells grow only up to the point where amino acids become limiting (Figure 79). In very low glucose concentrations local velocities almost completely overlap because amino acid limitation doesn't significantly affect growth anymore.

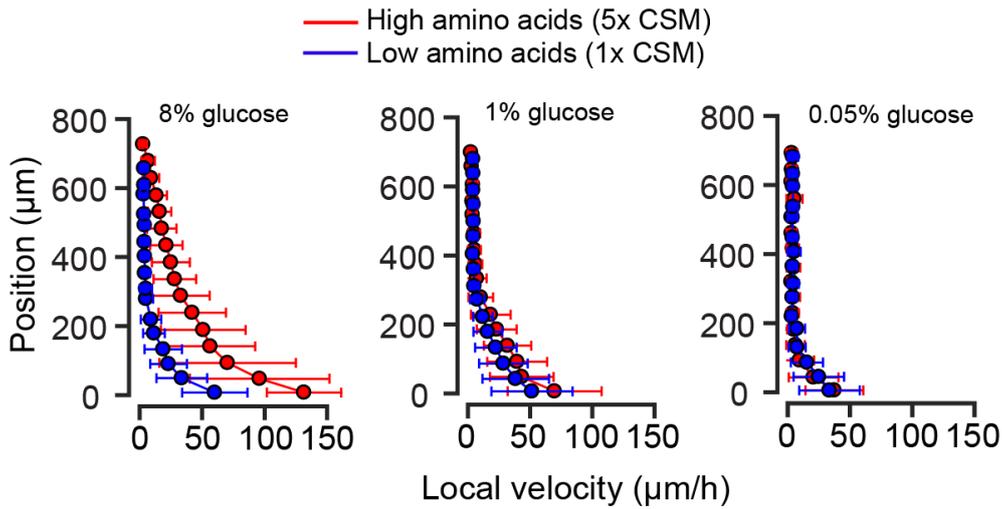


Figure 79 Evolution of local velocities within a colony in high (red) and low (blue) amino acid concentrations across different glucose concentrations. Each panel represents local velocity within a colony once cells fill out the cell chamber in the “Yeast Machine”. Depending on the starting glucose and amino acid concentrations, but also diffusion and uptake rates which evolve as a function of local nutrient concentration, the number of cell layers that grow and their growth rates are set.

To get a better insight into the effects of amino acid limitation and its role in colony self-organization and differentiation, we looked at the gene expression of genes we previously characterized in high amino acid concentrations, where no amino acid limitation takes place and growth is mostly limited by glucose availability in the local microenvironment. We used glucose transporter HXT7 as a marker of glucose concentration since it reaches the maximum expression in low glucose concentrations with a peak that should correspond to 0.0156% (0.86 mM) glucose concentration.

So, what could we expect to see if there is an amino acid limitation and cells stop to grow once there is no amino acids within a colony? A reasonable hypothesis is that glucose uptake will be diminished in cells that do not have access to amino acids and do not grow because the translation rates of proteins will be significantly impaired in amino acid starvation and cells will not consume glucose in large quantities since they are not growing. If this is true, we should observe a shift in glucose concentration gradient within a colony in parts where amino acids are limiting. Since cells do not uptake/consume glucose as much as when they have access to amino acids, there should be an increase of glucose concentration in parts that are not growing due to amino acid limitation, compared to when there is no

amino acid limitation. Conversely, parts of the colony closer to the nutrient source, that are below the point of amino acid limitation in low amino acids (1x CSM), should show no significant difference in glucose concentration gradient when compared to high amino acids (5x CSM) since they have access to amino acids in both cases. In terms of HXT7 expression this should mean that the peak of expression should be at similar positions in lower glucose concentrations when we grow cells both in low and high amino acid concentrations, but the peak of expression should be shifted deeper in the colony in low amino acids since cells do not uptake as much glucose above the point of amino acid depletion. A peak shifted deeper in the colony, further away from the glucose source, means that the position of low glucose concentration is shifted.

When we compare experiments in low and high amino acids across almost a 1000-fold range of glucose concentrations, what we stated in our hypothesis seems to be correct. We observe a very similar pattern of expression in low glucose concentrations and a completely different pattern in high glucose concentrations (Figure 80). A paradigmatic example of the shift in glucose concentration can be seen in 2% (111 mM) and 4% (222 mM) glucose concentrations. While in high amino acid concentrations the peak reaches 485  $\mu\text{m}$  in 2% glucose and 726  $\mu\text{m}$  in 4% glucose, in low amino acid concentrations the peak reaches 629  $\mu\text{m}$  in 2% glucose and it doesn't appear at all in 4% glucose concentrations along the whole length of a colony. Moreover, if we look at the expression intensity in 2% glucose, we can see that it is around 50% lower when amino acids are limiting compared to when amino acids are abundant.

If we look at the peaks of maximum expression only and compare their position as a function of starting glucose concentration, we see an overlap in low glucose concentrations in both high and low amino acids, followed by the start of separation in 0.5% and 1% glucose, and then a complete separation in 2% glucose and above (Figure 81). In high amino acids the peak of maximum expression doesn't increase linearly with the increase of glucose concentration, it seems to slow down, probably due to underlying complex interplay between diffusion, glucose uptake rates, and starting glucose concentrations. However, in low amino acids the position of the peak seems to evolve linearly with the increase of glucose concentration.

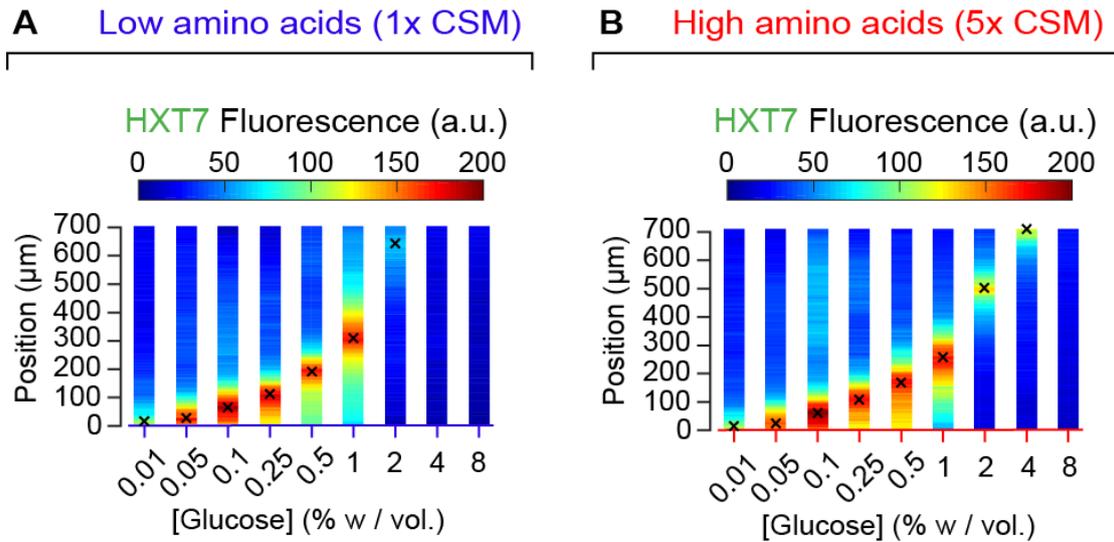


Figure 80 Gene expression landscape of the glucose transporter HXT7 in different glucose and amino acid concentrations. Position 0 µm is the position of the glucose source. (A) Low starting amino acid concentration (1x CSM). (B) High starting amino acid concentration (5x CSM). Black x represents the peak of expression. Fluorescence values represent measured values after the background is removed. Additional figures can be found here (Figure 106, Figure 101).

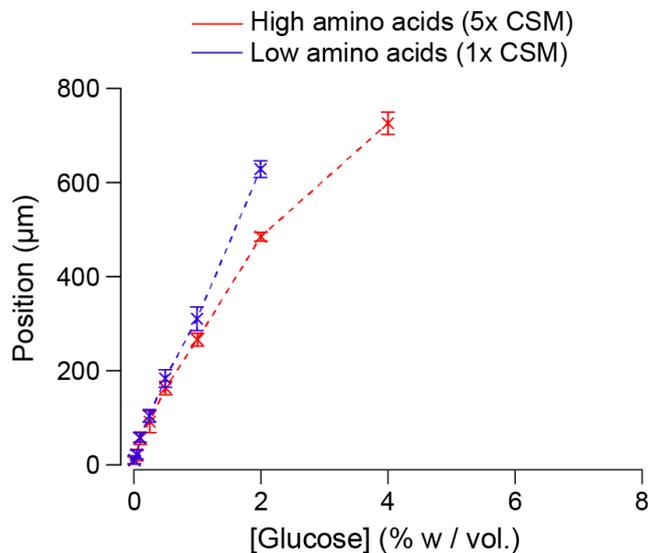


Figure 81 Peak of expression of glucose transporter HXT7 in high (red) and low (blue) amino acid concentrations. As starting glucose concentration increases, the peak of maximum expression moves deeper into the colony. Since cells uptake less glucose from the environment after the amino acid limitation is reached in low amino acids, the peak of maximum expression starts to separate when compared to high starting amino acid concentrations.

Glucose transporter HXT1 probably gives the clearest example of the position of amino acid limitation. HXT1 is expressed in high glucose concentrations. When we supply to a colony very high concentration of glucose, for example 8% (444 mM),

and when there is no amino acid limitation, we see the expression of HXT1 everywhere in a colony. In contrast to that, once amino acids become limiting but there is enough glucose to induce the gene expression in the whole colony in 8% glucose, there is a clear cut off of the expression due to the diminished translation rates of proteins in cells in amino acid starvation (Figure 82). We can also observe such clear cut off in 4% and 6% glucose too when compared between the two amino acid concentrations, and it appears at position around 300  $\mu\text{m}$  away from the nutrients source. This is a very similar distance of the peak of HXT7 expression in 1% glucose when there is no amino acid limitation.

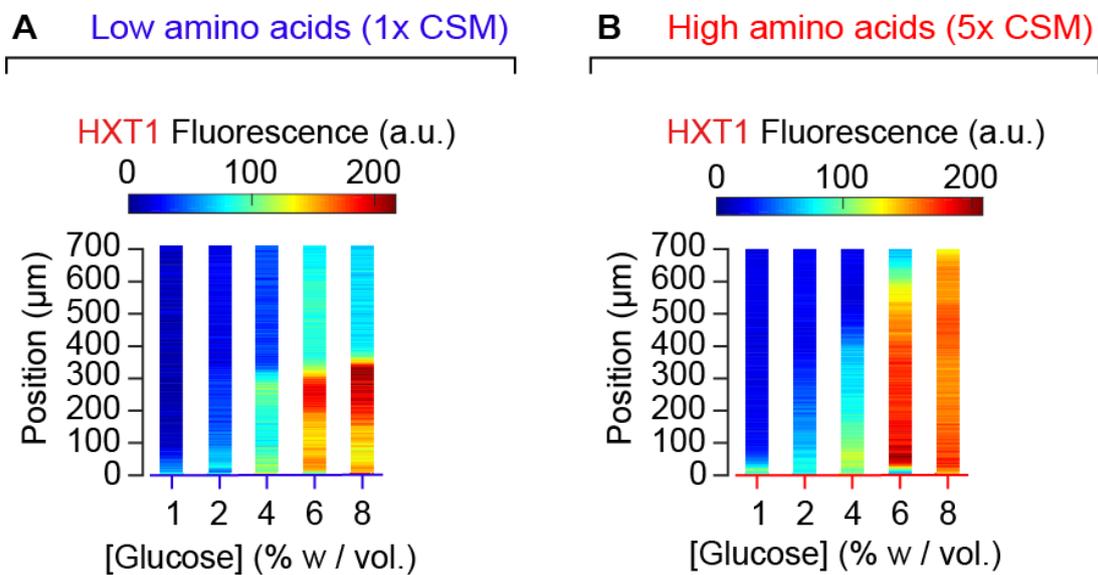


Figure 82 Gene expression landscape of the glucose transporter HXT1 in different glucose and amino acid concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (A) Low starting amino acid concentration (1x CSM). (B) High starting amino acid concentration (5x CSM). Additional figures can be found here (Figure 107, Figure 100).

When we look at the two genes with opposite effects, PDC1 that is overexpressed during fermentation in higher glucose concentrations and SDH2 that is overexpressed in low or no glucose concentrations during respiration, we can see how differentiation between the fermenting and the respiring subpopulation is different in low amino acid conditions compared to high amino acid conditions (Figure 83). In high amino acids, differentiation between the two population is clearest in 1% starting glucose concentration. PDC1 is overexpressed in the first part of the colony where glucose is high enough to induce fermentation and SDH2 is

overexpressed in the second part of the colony where glucose is low enough or absent which induces a switch to respiration. However, in low amino acids the switch between these two modes of metabolism is not as drastically clear. While the first part of the colony maintains high expression of PDC1 since there is enough glucose and amino acids in that part, in the second part of the colony SDH2 is up to three times less expressed as in high amino acids, probably because of the lower translation rates caused by amino acid starvation.

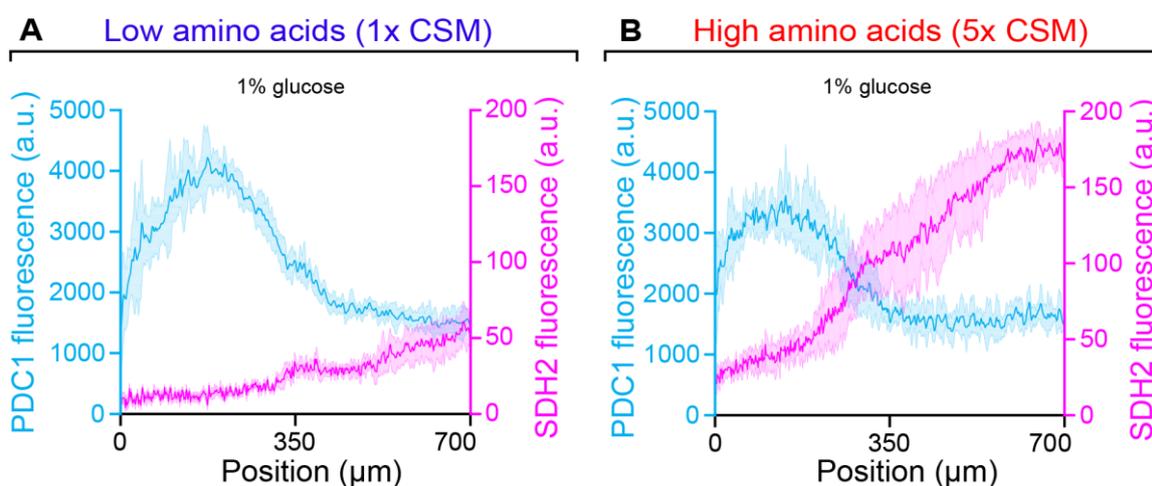


Figure 83 Gene expression of pyruvate decarboxylase PDC1 and succinate dehydrogenase SDH2 in low and high amino acid concentrations in 1% glucose (55.5 mM). Position 0 μm is the position of the nutrient source. (A) Low starting amino acid concentration (1x CSM). (B) High starting amino acid concentration (5x CSM).

The most peculiar is the case of glucose transporter HXT5. It is the glucose transporter that is thought to be expressed when cells enter growth arrest and its regulation mechanism is supposedly not directly affected by the Snf3/Rgt2 signalling pathway that regulates other major glucose transporters (independent of induction by low or high glucose concentrations), but is rather coregulated by Snf1/AMPK, Gpr1/cAMP/PKA and Pho85/Plc6/Plc7 signalling pathways [221]. It is worth noting that the exact mechanisms of HXT5 regulation are still not completely understood. HXT5 is also a transporter that lead to some confusion in the discovery of its regulation mechanisms because at first researchers thought that HXT5 is repressed by glucose since they saw its induction upon glucose depletion, during growth on non-fermentable carbon sources and during sporulation [222]. However,

it was later discovered that HXT5 induction signalling pathway is independent of extracellular glucose concentrations and that it depends on the growth rates of cells [223]. Researchers noticed a correlation between HXT5 expression and slow growth on other carbon sources, but more importantly they were able to control the growth rates in nitrogen-limited continuous cultures independently of glucose. HXT5 was expressed at low growth rates but not at high growth rates even though glucose was present at a concentration of 200 mM (~3.6%) across all tested growth rates. It has also been shown that HXT5 expression is induced by high temperature and osmotic shocks by NaCl and sorbitol [224], [225], and that its regulation is mediated by stress response elements (STRE), post-diauxic shift element (PDS) and Hap2/3/4/5p elements in the promoter region [203].

Based on this we expected to see induction of expression of HXT5 once glucose or amino acids become limiting with probable influence of amino acid limitation on expression levels of HXT5 compared to the case when there is no amino acid limitation. As it has been discussed earlier, in high amino acids the expression of HXT5 follows a relatively straightforward logic. Once the glucose concentration becomes limiting, cells enter into growth arrest and expression of HXT5 is induced. This leads to differentiation between roughly two subpopulations, one that grows and doesn't express HXT5 and the one that doesn't grow but does express HXT5. As starting glucose concentration is increased, the transition between two subpopulations happens further away from the glucose source as a product of changed glucose availability in the local microenvironment.

However, when we fed the colony with low amino acids, the first surprising expression pattern happened in 0.1% (5.5 mM) glucose concentration (Figure 84). The expression of HXT5 was almost twice as high in amino acid limitation than in amino acid abundance, although the expression patterns of other genes usually showed a significant decrease in the expression in amino acid limitation probably caused by diminished protein translation rates. Based on this unusual result, we expected to see increased levels of HXT5 expression in amino acid depleted parts of the colony when we increase glucose concentration, with the difference at the parts close to the nutrient source where we expected that the availability of glucose and high growth rates will inhibit HXT5 expression. Indeed, HXT5 repression followed the predicted behaviour but its induction in the amino acid depleted parts of the colony did not. In 1% glucose, the expression levels of HXT5 were slightly lower in low amino acids (1x CSM) compared to high amino acids (5x CSM), at the back of the colony. The unusual thing in this case, when we compare 0.1% and 1% glucose in low amino acids at the back of the colony, is that cells in this part of the colony are

not growing and are both in glucose and amino acid deprivation but still show more than one-fold difference in expression. Therefore, we think that there must be something in the environment of 0.1% and 1% glucose cases that causes either induction or repression, which should neither be glucose or amino acids. Although, there is a slight possibility that in 0.1% glucose conditions, amino acids are not depleted as fast as in 1% glucose conditions since less cells are growing due to glucose limitation and maybe consuming less amino acids, and it is definitely something worth checking.

In the 2% and 4% glucose case we enter into a slightly different regime of amino acid and glucose interplay. Amino acids are probably depleted after about 300  $\mu\text{m}$ , but glucose is not (in contrast to 1% glucose case where glucose should be also depleted after about 300  $\mu\text{m}$ ). The first thing that can be noticed in 2% and 4% glucose case is that the expression at the back of the colony is even more repressed in low amino acids compared to 0.1% and even 1% glucose case. If we take a closer look at 2% glucose in low amino acids we can see appearance of two “steps”, one at around 300  $\mu\text{m}$  probably caused by amino acid depletion and subsequent growth arrest, and a more interesting, slight but noticeable, increase in 600-700  $\mu\text{m}$  area. This is exactly the area where HXT7 expression peaks in 2% glucose condition in low amino acids (Figure 80 A, Figure 106), meaning that in this part glucose concentration is very low and tends to depletion. Finally, in 4% glucose in low amino acids there is a slight increase at around 300  $\mu\text{m}$  position but expression at the back is the lowest compared to all other glucose concentrations. In 4% glucose in high amino acids there is a slight increase at the end of the colony which corresponds exactly to the peak of expression of HXT7 in the same conditions.

There are two things I want to point out based on the peculiar case of glucose transporter HXT5. The first one is that the “Yeast Machine” microfluidic device can be a convenient tool to observe differences in cell behaviour across all concentrations of a specific nutrient, but also to explore combinatorial influence and interplay of two or more nutrient gradients. As it was possible here to induce growth arrest through amino acid depletion, but concentrations of glucose could be varied. This is possible thanks to the self-organization of single cells into colonies and emergence of long-range metabolic interactions. Moreover, it is closer to what often happens in nature, formation of heterogeneous environments and not homogeneous ones.

The second thing I want to point out is that it is possible that both publications, one that claimed that HXT5 is repressed by glucose and the other one

that claimed that HXT5 is induced by growth arrest, are actually right. Although, in the first publication they jumped to conclusion of glucose repression too fast and the second publication had a sounder approach by limiting the growth through nitrogen limitation and keeping glucose in the media, its flaw might be that they kept a constant glucose concentration. I suggest here that there is a possibility that HXT5 is indeed both induced by growth arrest but also repressed by glucose in dose dependant manner. I think it would be worth revisiting the experiment in nitrogen-limited continuous cultures, but this time also vary the glucose concentration.

**HXT5 gene expression in low amino acids (1x CSM) and high amino acids (5x CSM)**

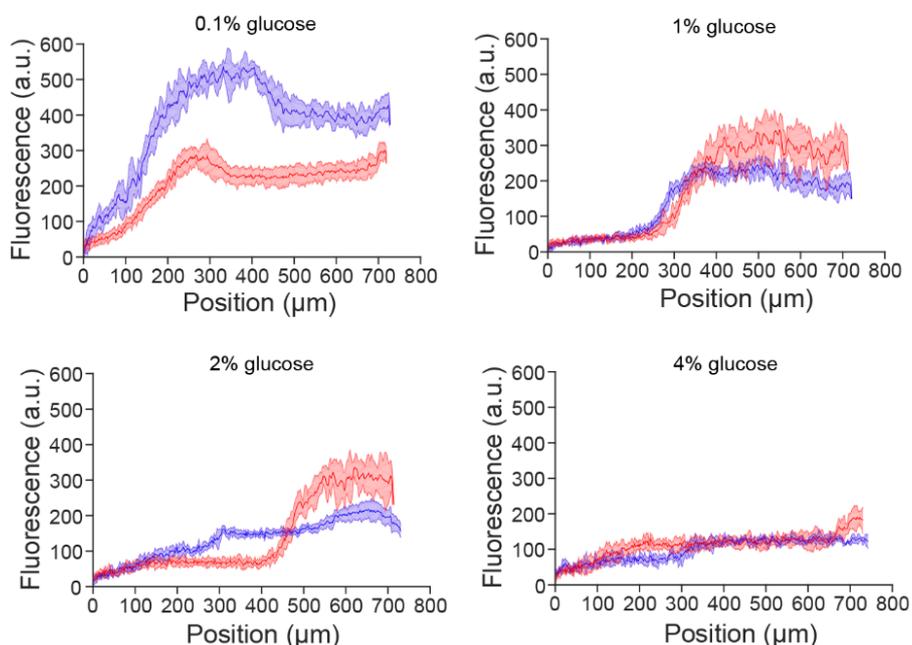


Figure 84 Gene expression of glucose transporter HXT5 in low and high amino acid concentrations in 0.1% (5.5 mM), 1% (55.5 mM), 2% (111 mM) and 4% (222 mM). Position 0 µm is the position of the nutrient source.

Here, we demonstrated that other nutrients form gradients in the colony too. Moreover, they can be complemented with the control of other nutrient gradients in order to get interesting gene expression patterns or even decoupling of growth from glucose concentrations. In nature, probably very often more than one nutrient is limited so we could use our device to simulate such nutrient scarce environment and see the adaptation by cells in real time.

## Impact of deletion of glucose transporters

Tinkering with the cell's genome inevitably leads to changes of its physiology. Nevertheless, the changes that we make, e.g. gene deletions, can lead to simplifying a complex problem or to a discovery of the role of a certain gene in a complex network of interactions. In the late 1980s and 1990s a significant effort was made to understand the role of glucose transporters in overall yeast physiology. The goal of one of those efforts was to create a strain that could not uptake glucose from its environment. Deletion of any single glucose transporter alone did not lead to any observable difference in growth phenotypes nor to significant changes in glucose uptake kinetics. Reifenberger constructed a strain that had seven glucose transporters deleted, HXT1-7, widely known as "*hxt-null*" mutant strain [92]. She concluded that this mutant was "*nearly unable to metabolize glucose*", and that individually recovered HXT 1, 2, 3, 4, 6 and 7 genes can use glucose "*at considerable rates*". More precisely, "*hxt-null*" strain achieved 10% growth rate compared to the wild type strain, and HXT 1, 2, 3, 4, 6 and 7 genes achieved 34%-98% growth rate and 24%-96% glucose consumption rate compared to the wild type strain when grown in rich YPD + 1% glucose media. Individually recovered HXT7 strain, for example, showed 90% growth rate and 87% glucose consumption rate compared to the wild type in 1% glucose. Reifenberger concluded that "*growth of the HXT7 strain was almost like that of the wild type on 0.1% or 1% glucose, but higher glucose concentrations were inhibitory to some degree*". In a follow-up study to determine the kinetic parameters of individual HXT1-7 genes, she concluded that glucose uptake was below the detection level in "*hxt-null*" strain, but each individual transporter was not essential for viability or growth on glucose [91]. With the advances in yeast genome sequencing a more complete picture of glucose transport was established, as it was determined that yeast *S. cerevisiae* has at least 20 glucose transporters HXT1 to HXT17, SNF3, RGT2, and GAL2 [88]. Subsequently, it has been discovered that SNF3 and RGT2 are very similar to glucose transporters but are probably not able to transport glucose, but rather they serve as extracellular glucose sensors which trigger an intracellular signalling cascade in response to extracellular glucose [94]. As far as other glucose transporters are concerned, HXT8-17, it has been concluded that they do not affect the glucose uptake significantly, probably as a consequence of their reduced expression level, with an important note, in common laboratory conditions [90], [95]. Although they still remain poorly studied, HXT8-17 have been shown to be expressed in different environmental conditions like

hypoxia, alkaline stress, osmotic stress, copper limitation, and in presence of non-fermentable carbon sources, as well as to be transporters of other molecules like polyols [96], [106], [226].

Eckhard Boles' group proceeded with deletion of all glucose transporters as well as three genes that are a part of maltose permease subfamily which showed to mediate residual glucose uptake [93]. With the deletion of 21 genes they finally concluded that glucose consumption and transport was completely abolished. This widely used strain is known as EBY.VW4000 and strain EBY.VW5000 has additional deletions of glucose sensors SNF3 and RGT2. Recently, a complete sequencing of EBY.VW4000 genome was published and it was concluded that 16 successive deletion rounds with the *LoxP*/*Cre* recombination system introduced gene deletions and chromosomal rearrangements which could affect response of that strain in unforeseen ways [227]. Therefore, the conclusions about the response of that strain, especially when compared to the "wild type" background strain CEN.PK2-1C (which already has HXT17 deleted), should be taken with caution.

We wanted to reduce the complexity of the glucose uptake system to one glucose transporter only, so we were kindly provided by Eckhard Boles the strains CEN.PK2-1C, EBY.VW4000, and EBY.VW5000. Based on these strains, a large effort could be made to reintroduce back glucose transporters one by one for characterization in colonies, as well as making additional deletions in key glucose regulatory genes, but with the limited timeframe each thesis is subjected to, we opted for the recovery of glucose transporter HXT7.

As already mentioned earlier, there is a significant variability between *S. cerevisiae* strains [217]. So far, we used S288c background strain and there are more than 20,000 single-nucleotide polymorphism differences between this strain and CEN.PK strain, in addition to 83 genes that are absent from S288c strain. This is why we did not want to use S288c strain for comparison, but rather use the original background strain. We first had to characterize the response of the background strain CEN.PK2-1C, in which we endogenously tagged HXT7 with yeast enhanced GFP. Then we used this construct to recover HXT7::yEGFP in EBY.VW4000 and EBY.VW5000 strains at the native genomic locus. From now on I will refer to the CEN.PK2-1C strain with endogenously tagged HXT7 as the wild type (WT), EBY.VW4000 with recovered HXT7 as HXT7 only strain, and EBY.VW5000 with recovered HXT7 as HXT7 only, no sensor strain.

When we compare the two different backgrounds, there is not much difference between their response within a colony, especially in terms of the peak of maximum expression (Figure 85 A, B, C). There is a big difference in the level of fluorescence, but this can probably be attributed to the different versions of GFP these strains are tagged with. When we look at the colony front velocity, we do see a difference in higher glucose concentrations, as S288c reaches a plateau at around 116  $\mu\text{m}/\text{h}$  and CEN.PK reaches a plateau at around 130  $\mu\text{m}/\text{h}$ . This difference might be caused by difference in growth rates between two strains and slight differences in the glucose gradient evolution between glucose source and the peak of expression.

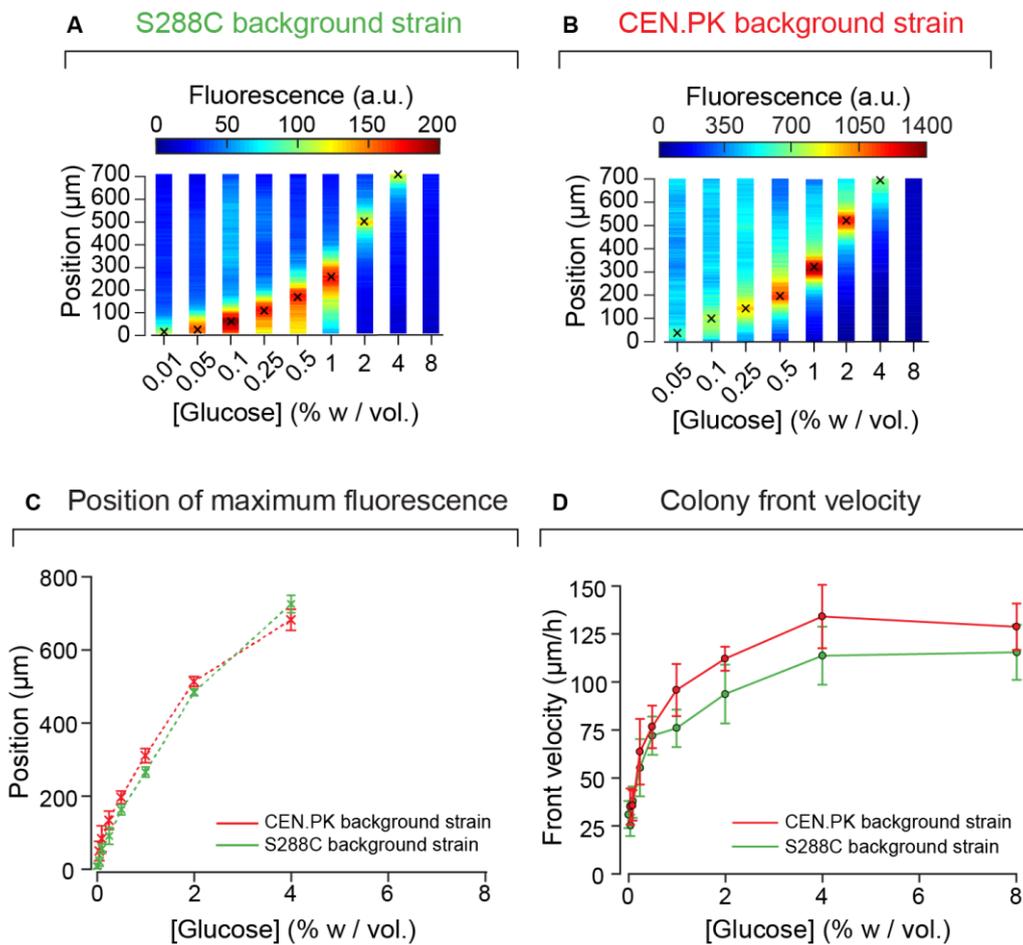


Figure 85 Comparison between S288c and CEN.PK strains. (A) Gene expression of glucose transporter HXT7 in different glucose concentrations in S288c strain. (B) Gene expression of glucose transporter HXT7 in different glucose concentrations in CEN.PK strain. (C) Positions of peaks of maximum HXT7 expression in different glucose concentrations. Position 0  $\mu\text{m}$  is the position of the glucose source. (D) Colony front velocity as a function of glucose concentration. Additional figures can be found here (Figure 101, Figure 108).

What happens when we delete all glucose transporters but one, HXT7? A simple hypothesis would state that since there is only one transporter expressed and not up to 20 of them, cells will uptake less glucose and this will reflect in the expression patterns of HXT7 within colony, especially in the position of the peak of expression which should correspond to the concentration of 0.0156% (0.86 mM). First, let us look at the expression of HXT7 in HXT7 only strain. It looks similar to the expression pattern of the background strain with all glucose transporters (except HXT17). We can see the peaks of expression and their positioning further away from the glucose source as starting glucose concentration is increased (Figure 86). The emergence of peaks of expression is not that surprising since HXT7 is expressed in low glucose concentration, but it is surprising that the expression pattern looks so similar to the wild type strain.

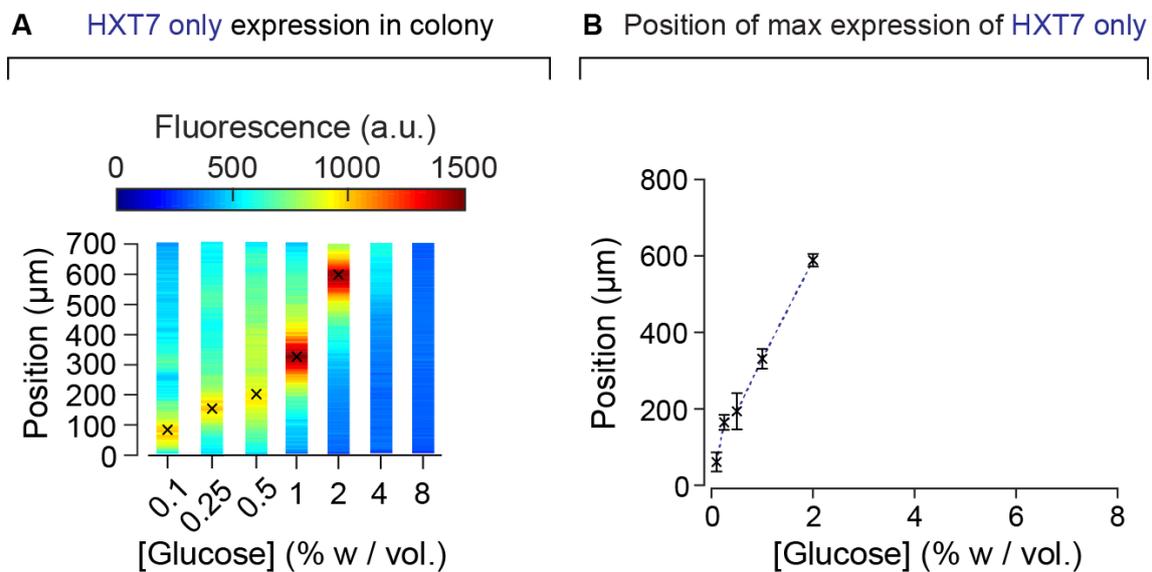


Figure 86 Gene expression landscape of the glucose transporter HXT7 in different glucose concentrations in HXT7 only strain. Position 0 µm is the position of the glucose source. (A) In colonies, the peak like expression pattern moves deeper inside colony as starting glucose concentration is increased. Black x represents the peak of expression. Fluorescence values represent measured values after the background is removed. (B) Peak of expression of glucose transporter HXT7. Additional figure can be found here (Figure 109).

To better understand what is going on, we need to compare the expression patterns directly between the wild type and HXT7 only strain across a range of glucose concentrations (Figure 87). In concentrations of glucose up to 1% (55.5 mM) the peak of expression seems to emerge at very similar positions. At 2% (111 mM)

glucose concentration the peaks start to separate, HXT7 only strain expresses it further away from the glucose source (Figure 88). This should happen when less glucose is taken up by the cells within a colony compared to the wild type, as stated in our hypothesis at the beginning. The surprising thing here is that the separation of the peaks happened only at such a high starting glucose concentration. However, there is one significant difference that is probably the cause of this behaviour within a colony. If we focus on the parts closest to the glucose source, we can see that at 0.5% (27.5 mM) starting glucose concentration, the levels of expression start to be significantly different. The same can be seen in 1%, 2%, 4%, and especially in 8% glucose. Although HXT7 should be repressed in high glucose concentrations, HXT7 only strain expresses HXT7 at around 25% of maximum expression level in high glucose concentrations. It is highly interesting that cells can “compensate” the uptake of glucose from the environment by all the other glucose transporters by expressing HXT7 at around 25% of its maximum expression. This “compensation” is especially visible at 1% starting glucose concentration, but indeed does slow down at 2% and 4% glucose, whereas we cannot assess it for 8% glucose since we cannot measure the glucose concentration gradient from these profiles and we would need a longer microfluidic device to see the emergence of peaks. This observation also raises a question of the need to have up to 20 glucose transporters (or even only 7 major ones) when one is already enough to secure glucose uptake for growth of the colony.

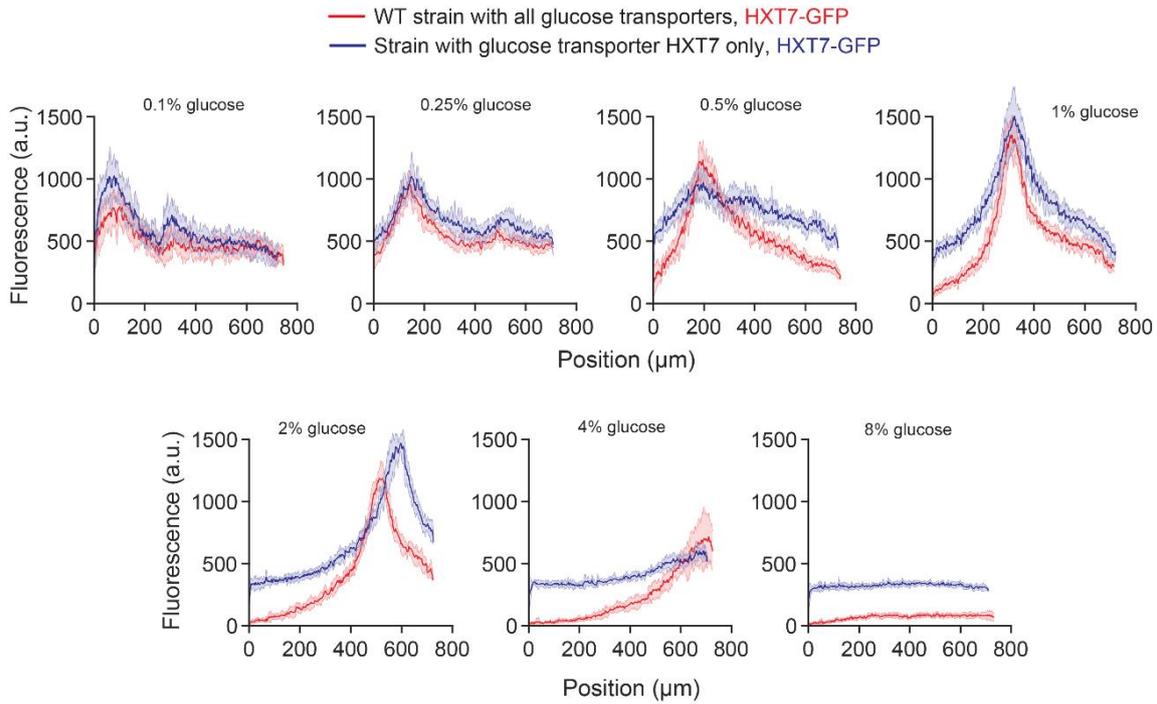


Figure 87 Comparison of gene expression of glucose transporter HXT7 in different glucose concentrations between the wild type strain that has all glucose transporters (except HXT17) and the strain that has only HXT7.

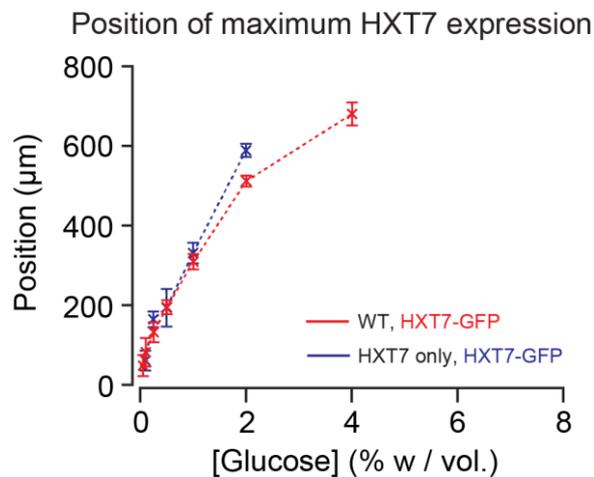


Figure 88 Comparison of positions of maximum expression of glucose transporter HXT7 in different glucose concentrations between the wild type strain that has all glucose transporters (except HXT17) and the strain that has only HXT7. The peak of expression overlaps up to the 1% (55.5 mM) glucose concentration and starts to diverge in 2% (111 mM) starting glucose concentration.

Researchers have previously shown that wild type levels of glucose transport are required for normal glucose repression of different genes, like the ones that take part in respiration, gluconeogenesis, and utilization of other carbon sources [91],

[228]–[231]. Kruckeberg’s group has also reported a similar finding for HXT7 [232]. Namely, they used Reifenberger’s “*hxt-null*” strain, which has major glucose transporters HXT1-7 deleted, to recover HXT7 and thus make “HXT7-only” strain. In this strain HXT7 was derepressed in high glucose concentrations, although it is tightly repressed in the wild type strains. They concluded that this must be happening due to relief of glucose repression as a product of reduced transport capacity and intracellular concentrations of glucose.

HXT7 only strain that we made is based on a different background that has all HXTs deleted. We quantified the expression of HXT7 in our HXT7 only strain by growing cells in batch culture at different glucose concentrations and then measuring expression using flow cytometry as described previously in this manuscript. In agreement with our experiments in a colony and experiments from Kruckeberg’s group, HXT7 in HXT7 only strain is indeed derepressed at high glucose concentrations to a level of about 35% of the maximum expression which occurs in 0.0156% (0.86 mM) glucose (Figure 89). The CEN.PK wild type strain shows a similar pattern of expression like S288c wild type strain, with a difference in the level of fluorescence intensity probably in most part due to the different type of GFP used.

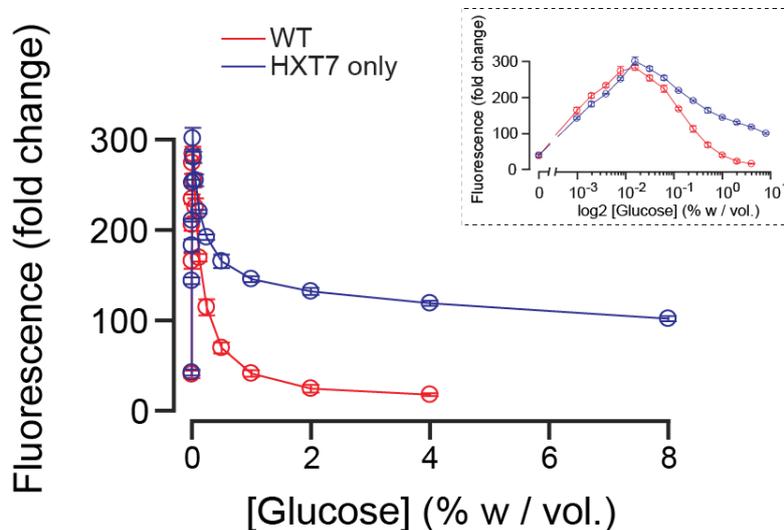


Figure 89 Gene expression of glucose transporter HXT7 as a function of glucose concentration in the wild type strain that has all glucose transporters (except HXT17) and the strain that has HXT7 only. HXT7 only strain shows derepression of HXT7 repression in high glucose concentrations. (main figure: linear x-axis; insert: log2 x-axis)

There are several sources which could cause HXT7 derepression in HXT7 only strain. The first one is promoter length. It is not completely clear what constitutes a full HXT7 promoter. There is more than 3 kb space between the start of HXT7 gene and the end of HXT6 gene. Researchers usually use around 1 kb long promoter and this is what I did too [233]. Ye deleted parts of the HXT7 promoter starting from around -1.2 kb and concluded that 1 kb should be considered as enough length for HXT7 promoter [234]. The major regulation sites appear to be somewhere around 500 bp of the promoter and scientists often use a truncated version that is only 391 bp long to get high constitutive expression without the influence of glucose concentration gradient [235]. The second possibility is that HXTs somehow interact with each other and the deletion of all of them but one kills this interaction and allows for derepression in high glucose to happen. The third possibility is that the derepression is caused by gene deletions and rearrangements that happened during rounds of deletion. Since derepression happens both in Reifenger's "*hxt-null*" strain (which needed much less rounds of cloning) and Boles' EBY.VW4000 strain, it is not likely that same mutations that could target glucose derepression happened. The fourth possibility, and my favourite one is that cells sense glucose influx level or flux through glycolysis so either this or glucose internal concentration influences the expression pattern of HXT7. In private correspondence with Eckhard Boles and Arthur Kruckeberg we agreed that this is likely what happens but that it still needs a formal demonstration after almost 20 years. The hypothesis is that there is a tension between HXT7 induction and HXT7 repression. Since cells import less glucose when they rely only on HXT7 compared to the wild type strain (because HXT7 gets saturated quickly in high glucose concentrations since it is a high-affinity transporter), cells have an impression that they are in low levels of glucose which would otherwise induce HXT7 expression in a wild type strain. It is also a question if this tension is a product of integration of extracellular glucose concentration signals and glucose influx, or glucose influx only. There is an interesting exception to HXT7 derepression, a strain for which the authors claim that is unique in this respect. This strain is a product of induced mutagenesis for biotechnological purposes and it retains derepression of HXT7 when glucose transport is similar to the wild type levels, achieved by adding HXT1 transporter to the otherwise HXT7 only strain [236].

By integrating some insight that we gained so far regarding the HXT7 only strain, that its growth rate is similar to the wild type strain, that glucose uptake is not completely abolished in high glucose concentrations, and that the peak of expression in colonies diverges at 2% glucose concentration or higher, we could

expect to see very little difference in colony front velocities between the wild type strain and HXT7 only strain. This is exactly what we see and while it is interesting in itself, it doesn't come as a surprise knowing things from previously published and our own research (Figure 90).

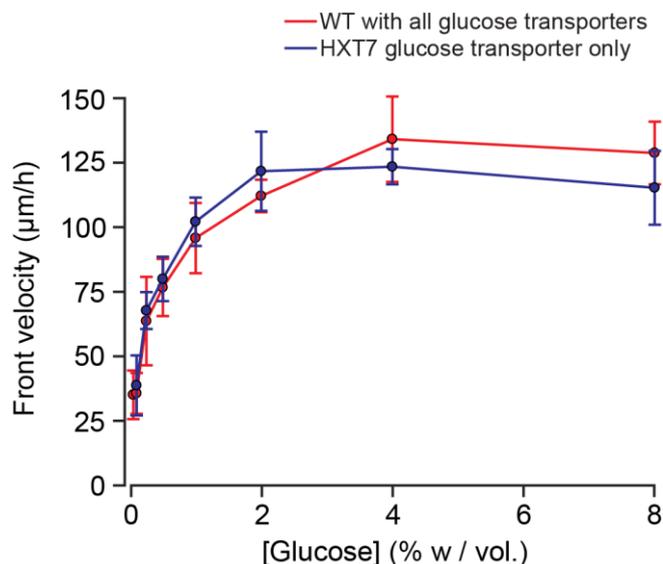


Figure 90 Colony front velocity as a function of starting glucose concentration in wild type (red) and HXT7 only (blue) strain.

It is a fact that many metabolic pathways go through significant rearrangements as a response to the external glucose concentration [71], [72]. However, it is under debate how that information is processed by a cell. Whether cells respond to external glucose concentration through glucose sensors SNF3 and RGT2, and/or they measure glucose influx through transporters, and/or they measure metabolic fluxes (through glycolysis, for example), and/or they measure intracellular glucose concentration, has been under conflicting reports. For example, while one group of scientists suggest that yeast can measure glucose or metabolic flux [231], [237], [238], the other group opposes that this is not possible [182], [228], [239]. On the other hand, in a recent publication by Barkai's group, they showed that volume growth of yeast is dominated by external glucose concentrations, while division rate is determined by glucose influx and that there is a tuning between those two parallel processes through an inherent feedback-dependent coupling [240]. Youk, however, demonstrated that growth rate landscape is set by external glucose perception and glucose import, not by their individual actions [182]. More specifically, growth rate could be inhibited by increasing extracellular glucose concentrations while keeping glucose influx

constant. When they deleted glucose sensors SNF3 and RGT2, a cell in 4% glucose acted as if it were in 0.06% glucose with intact sensors. A recent meta-analysis captured the complexity of this topic, but nevertheless researchers concluded that metabolic phenotype of yeast is regulated in a glycolytic flux-dependent manner and they suggested that the concentration of fructose-1,6 bisphosphate (FBP) and perhaps some other metabolites are flux-dependent which makes them good target as potential flux sensors [241]. This subject is far from being closed and with the advances in systems biology approaches like high-throughput analytical technologies such as metabolomics and fluxomics, we should obtain better answers.

Since glucose sensing is an important part of yeast response, we were interested to see how the lack of glucose sensors SNF3 and RGT2 will shape growth and gene expression of HXT7 only strain. In batch culture, HXT7 only strain with no sensors seems to express HXT7 at glucose derepression levels seen in HXT7 only strain with sensors between 0.0156% (0.86 mM) glucose and 8% glucose (444 mM). In glucose concentrations lower than 0.0156%, HXT7 expression falls down rapidly. Sensorless strain seems to be unable to capture the increase of HXT7 expression in low glucose concentrations. In colony, however, there is no systematic trend of HXT7 expression except a noticeable drop in expression in 8% starting glucose concentrations within the whole colony (Figure 91).

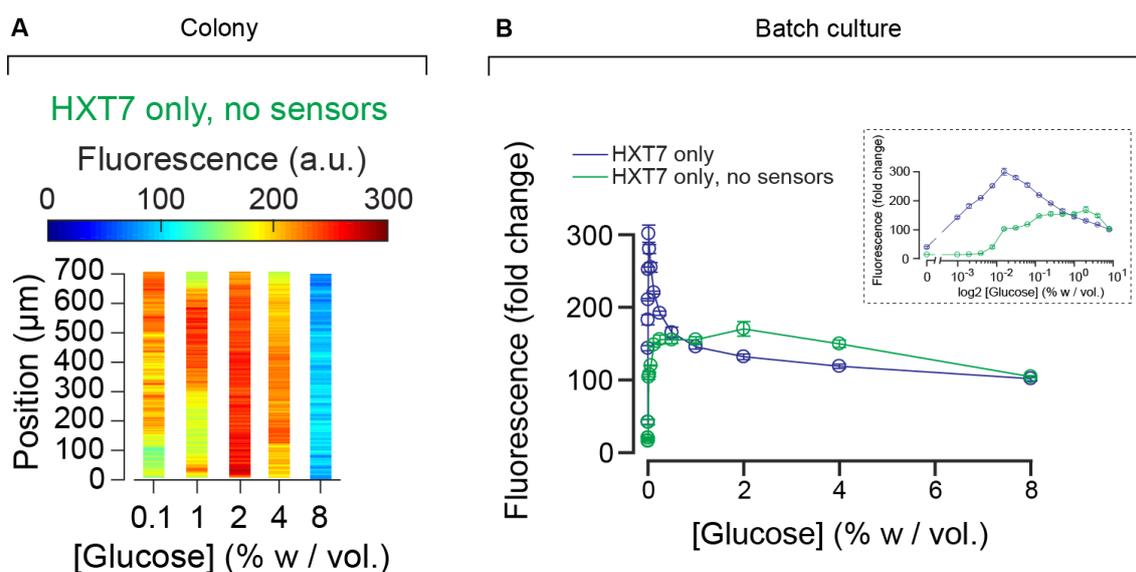


Figure 91 Gene expression landscape of the glucose transporter HXT7 in different glucose concentrations in strain with no glucose sensors and HXT7 only. Position 0  $\mu\text{m}$  is the position of the

glucose source. (A) In colonies, there is no systematic trend of expression. The expression is low within the whole colony in 8% starting glucose concentration. (B) Gene expression of glucose transporter HXT7 as a function of glucose concentration in the HXT7 only strain (blue) and HXT7 only strain with no sensors (green). (main figure: linear x-axis; insert: log<sub>2</sub> x-axis). Additional figure can be found here (Figure 110).

If we compare HXT7 only strain with sensors and HXT7 only strain without sensors in colony, we can see that peak like expression is completely abolished. However, HXT7 expression in sensorless strain is not completely abolished meaning that this strain is probably still capable of taking up glucose from the environment. In 8% glucose concentration sensorless strain exhibits the lowest expression and it seems to be derepressed less than the strain with sensors (Figure 92).

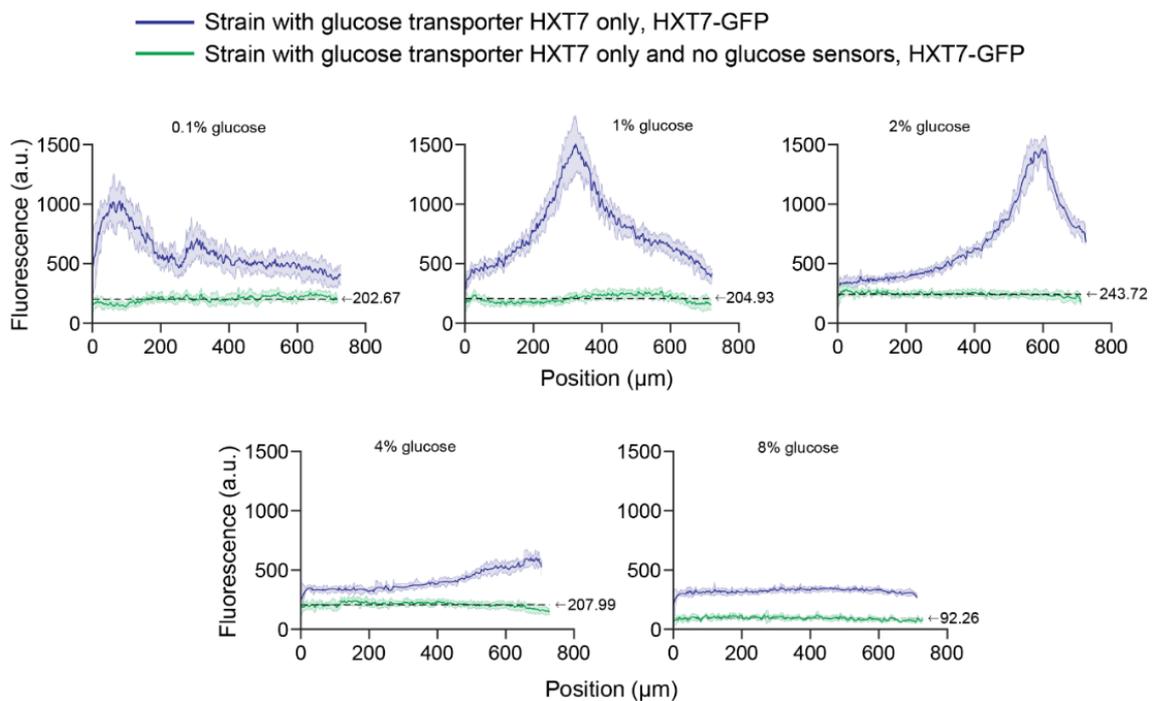


Figure 92 Comparison of gene expression of glucose transporter HXT7 in different glucose concentrations between the strain that has only HXT7 (blue) and HXT7 only strain with no sensors (green).

It is hard to predict growth of sensorless strain in the colony based on the HXT7 expression data. Looking at the colony front velocity we can see that in glucose concentrations of 0.1% and 1% the front velocity is very similar to the velocity of

the strain with sensors (and the wild type strain too). Beyond 2% starting glucose concentration colony velocity starts to fall, reaching similar levels in 8% glucose as in 0.1% glucose. Interestingly, Youk also saw the quenching of growth rate in sensorless strain in batch cultures in high glucose concentrations.

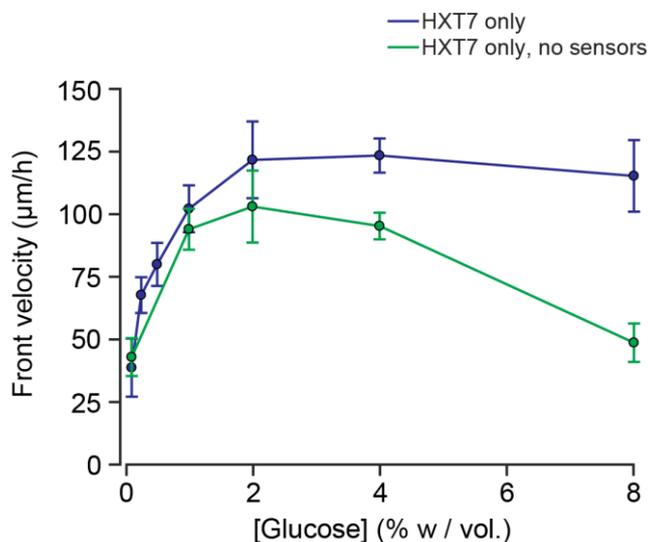


Figure 93 Colony front velocity as a function of starting glucose concentration in the strain that has only HXT7 (blue) and HXT7 only strain with no sensors (green).

Interplay between different glucose signalling pathways is still an unresolved problem. It is especially unclear how glucose sensors influence the growth rate and what is the role of glucose transporters in it. It is indeed a complex network of many interactions and whenever we try to intervene in the normal cell physiology, either by making gene deletions or integrating some kind of synthetic control, we disrupt the common processes and it becomes hard determining what is a cause and what is a consequence. We have seen that, surprisingly, glucose transporter HXT7 is derepressed and this led to almost identical expression patterns up to 1% glucose concentration and almost identical front velocities. When we removed the sensors, the expression pattern of HXT7 was completely changed but cells still managed to grow well, as a colony, at comparable velocities as the wild type and the HXT7 only strain. Only in very high glucose, the strain without sensors grew less well. Indeed, in 8% glucose in sensorless strain HXT7 is expressed at lower levels in colony and this might be the reason why it grew slower. But it is hard to be completely decisive about this conclusion as some other mechanisms might have caused the growth to stall.

## **Perspectives**

If we consider all our results in totality, I think we have been able to convincingly demonstrate that single cells indeed do exhibit emerging properties when they come together and “self-inflict” nutrient gradients on themselves. This principle looks quite simple, it is a mere uptake of glucose, but we have seen how complex regulation between sensing, signalling, uptake, and growth can be. It is still an outstanding question why have the yeast cells evolved so many glucose transporters, what are the underlying mechanisms that would lead to this. Maybe if we would test many different, nutrient rich and nutrient poor, conditions in the Yeast Machine, we would get a more comprehensive picture of the complex “hxtology”.

There are many side projects that could evolve from this project. Every time I read in a paper or in a review that we need to move closer to the natural environment I think about, well, why not try it in the “Mother Machine”. If I had to choose my first approach would probably be just to try to push the project as high throughput as possible. This is not because it is an easy way to get a lot of data (that might turn out to be insignificant anyway) but I truly believe that this is a great way to look at different phenomena via gene expression and growth quantification, in the whole range of nutrients with the added benefit that this is closer to the interactions in nature. If it was possible to independently load a lot of strains on one high-throughput chip we could map environment to phenotype interactions for a great number of genes. We could also screen the gene deletion libraries to map the interactions between environment and the lack of a specific gene. We were able to produce glucose and amino acid gradients, but there are many more nutrients that should be tested, either separately where everything is in excess but one nutrient or in combinations of different nutrient limitations. Some obvious candidates are nitrogen, phosphate, and oxygen limitations, utilization of other carbon sources both which can be fermented and which can only be respired. An interesting notion that could also be tested with this setup is a hierarchical use of different carbon sources. We can imagine, for example, mixes of glucose and galactose and see if the population close to the nutrient source will first preferentially deplete glucose and the one far away from the glucose source galactose. If this was to happen we would effectively have a phenotypic differentiation that is driven by the mix of carbohydrates in the media. Another important question connected to this is how much are cells within a colony feeding on their excreted by-products. For example,

do cells within the colony grow on the produced ethanol? If they do, where in the colony do they do it, how, and when? And what is the benefit they get from it.

One of the perspectives that is missing here is what happens when colonies are grown for a longer time. I hope I will be able to tackle that side of the story too until the defense so that I can include it in the final manuscript. But from the preliminary data we can roughly see three modes of growth and gene expression differentiation. One in lower glucose concentration, up to about 1% glucose, where patterns of expression and growth distance seem to be stable in time. The second in 2% glucose, and in part in 4% glucose, where we can observe the movement of the peak of HXT7 expression deeper in the colony along with the growth. This point is interesting to think about from an evolutionary point of view. My favourite scenario which is probably hard to prove is that yeast cells behave selfishly in low glucose, they “know” that glucose is low and they do not want to share it. But what if, when glucose is high enough, they do not selfishly take up as much glucose as they can but rather they share it with others, the glucose uptake downregulates in time to maximize the glucose penetration into the colony. Of course, this might not be an intentional behaviour but just the by-product of biophysical laws or limitations. The third time-dynamics scenario happens in very high glucose concentrations, like 4% and 8% glucose. Cells grow normally for some time but at one point a large portion of the colony stops to grow, which is also accompanied by large changes in gene expression. Usually, the cells closest to the nutrient continue to grow and overexpress genes, while cells at the back stop to grow and downregulate the gene expression. The most plausible scenario is that there is an accumulation of inhibitory by-products which are rapidly produced by the whole colony in such high glucose concentrations but at this point we do not know which exactly by-product or by-products cause that.

An outstanding question would also be how do colonies adapt to the dynamically changing starting glucose concentrations. These controlled fluctuations might mimic the nature even better as there are constant fluctuations of nutrients in the environment. We mostly focussed our inquiry to the constant starting nutrient concentration but dynamic control could be seamlessly implemented in the “Yeast Machine” system. This way cells that are starving at the back of the colony might periodically get nutrients, and cells at the front of the colony might periodically be depleted of nutrients. We could also couple our device with the optogenetic control and target only specific parts of the colony. This tool would give was an additional spatiotemporal control. For example, what would happen if we would induce nutrient uptake in only one specific part of the colony? With the dynamic change of

the environment we could also test possible bet-hedging strategies. Maybe a part of the starving cells at the back hedge their bets in expectation to incoming nutrients, so we could measure if this happens and how much cells benefit from it. We could also see if these strategies evolve in time within a colony and even the same cells within a colony. We did see some possible indications that a small fraction of the starving cells expresses glucose transporters that should otherwise be shut down.

One upgrade of the “Yeast Machine” could be superposition of nutrient gradients along the colony. This way we could impose nutrient gradients to the colony instead of letting the colony to “self-inflict” gradients to itself. It would be very interesting to see if by doing this we would get the same self-organization and differentiation patterns or not. It would also be very beneficial if we could somehow extract yeast in high enough numbers from different positions and then analyse the transcriptome with RNA-seq.

We also observed very high rates of death in very specific conditions, when both amino acids and glucose were limiting in the same area cells died rapidly and in large numbers. Maybe they could not cope with this transition between glucose and amino acids presence and absence which has triggered some kind of a conflict in cells and led to apoptosis.

Tolerance to stress, aging, sucrose utilisation and the emergence of cheaters within colonies are examples of even more interesting topics. A convenient fact is that yeast have not been extensively used to look at population level phenomena like bacteria have been due to the fact that they make biofilms, which is a completely different field on its own. A recent example of the emergence of population level phenomena in yeast is a paper from Markus Ralser’s lab where they looked at the “self-establishing communities” that were able to cooperatively exchange metabolites [218]. They inoculated on gel *S. cerevisiae* strain that had auxotrophic markers on plasmids. As cells were growing some of the plasmids got lost, so a colony was composed of yeast which were auxotrophic for a certain amino acid. However, they were able to grow because they used amino acids that were released in the environment by other yeast that were producing it, effectively generating a very heterogeneous colony. Interesting is that researchers tried to do this kind of complementation experiments in liquid cultures, but they never managed to make yeast complement each other and grow. However, in colony where they grow tightly together they complement each other.

I will finish the perspectives part with a project that I particularly like. It is not about yeast, but it is about tumours. It is an example of how very similar principles can be applied to other groups of cells, like cancer cells. Carlos Carmona-Fontaine and his colleagues studied how spatial structure emerges in tumour microenvironment due to Warburg effect (which is, as I mentioned before, similar to Crabtree effect in yeast) [242]. Tumour cells are grown in an *in vitro* microphysiological system named the metabolic microenvironment chamber (MEMIC). It consists of a chamber for cells and a slit that connects the chamber to media. Tumour cells consume nutrients and secrete waste products which effectively creates a gradient of nutrients inside the cell chamber. For example, oxygen gradients form along the tumour cell assembly and lactate accumulates at the back of the cell chamber (Figure 94). In my postdoc, I will work with Carlos to upgrade this device so that we can co-culture different types of cells and I hope I will be able to apply some knowledge gained through work on yeast to tumour cells. With this, I finish the main part of this thesis.

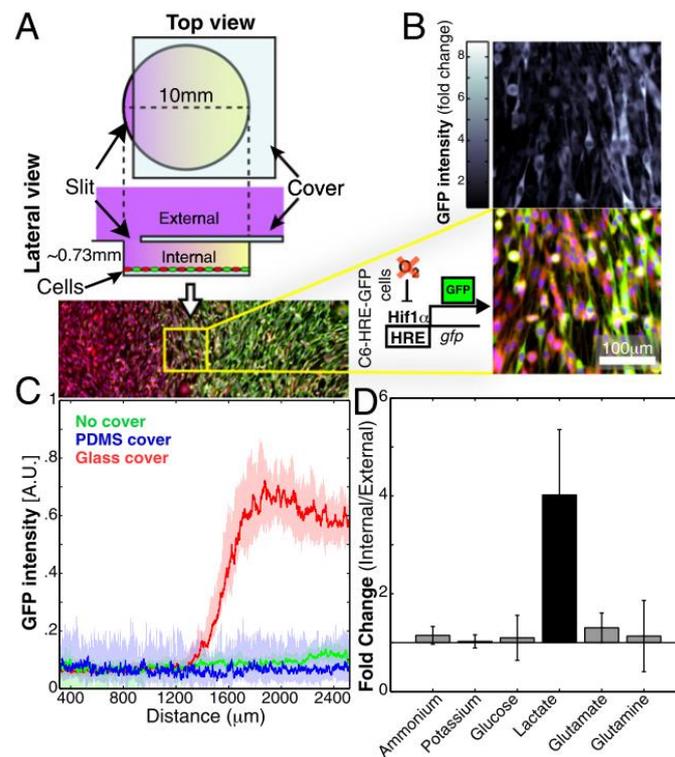


Figure 94 Emergence of local microenvironments in tumour cells. (A, B) Cells are grown in a dead-end cell chamber that is connected to media through a slit. As tumour cells grow, consume nutrients, and create waste products the environment becomes progressively short on oxygen. (C, D) Oxygen gradients are formed and lactate accumulates in the internal part of the cell chamber. Figure from [242].



# Appendix

## Protocol for the microfluidic device fabrication

We used a clean room at our university to microfabricate our own microfluidic devices. This allows us to experiment with soft lithography and microfabrication techniques so that we can design devices which will serve our experimental purpose best while being cost-efficient. During my PhD I went through 18 different designs until I found the one that was best suited for observing growth and differentiation of yeast colonies.

There are several techniques for the fabrication of microfluidic devices. We are using soft lithography and PDMS moulding. The first step is to design a photomask. We designed the photomask using L-Edit software (Tanner) and it was printed on a high-resolution glass substrate (Delta Mask) (Figure 95 A). The chrome mask serves to produce patterns on a silicon wafer that is used as a master mould for chip production (Figure 95 B).

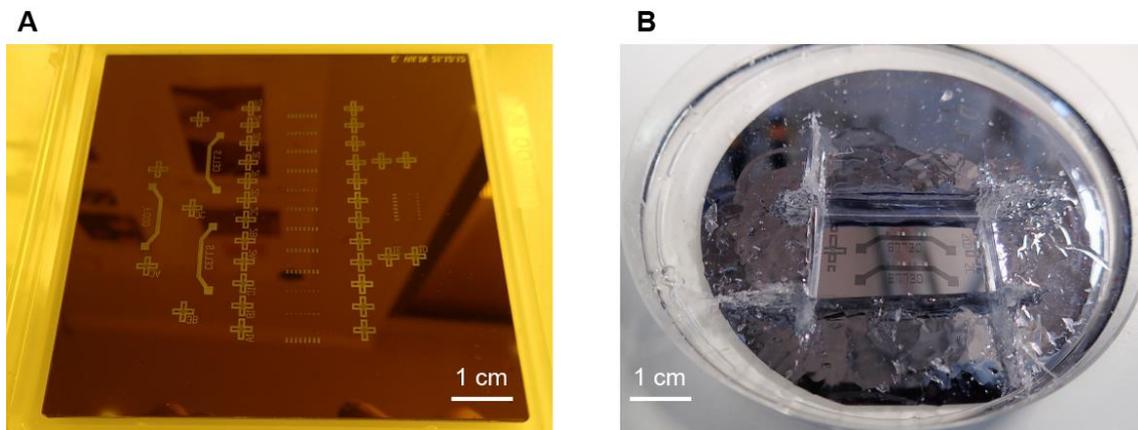


Figure 95 Mask and wafer for fabrication of the “Yeast Machine”. (A) A chrome mask with many different motives for microfluidic device fabrication. It contains chambers of different shapes, patterns, and purposes. Crosses are used for the alignment of different motives. (B) A silicon wafer that serves as a master mold for the fabrication of the “Yeast Machine”.

The protocol for making the master wafer is as follows (Figure 96):

1. The silicon wafer is prepared before its use. It is cleaned and its surface is activated with  $O_2$  plasma at 6 mbar for 6 minutes.
2. SU-8 2000 (MicroChem) epoxy-based resin of known thickness is spread on the silicon wafer. There are twelve variants of the SU-8 200 resin that

differ in their viscosities. With them we can make a resin layer of 0.5  $\mu\text{m}$  to 200  $\mu\text{m}$  thickness with a single coating process. Since the “Yeast Machine” is composed of two layers that overlap, one 4.5  $\mu\text{m}$  thick that serves for cell chambers and the other 25  $\mu\text{m}$  thick that serves for nutrient channel, we start with the thinner one. For this we used SU-8 2005 resin that was spread on the silicon wafer and then spun on a spin coater in two steps, 500 rpm for 10 seconds and 8000 rpm for 30 seconds.

3. Then we “soft-baked” it for 2 min at 95°C on a levelled hot plate.
4. Epoxy-based resin is photosensitive and it solidifies through cross-linking once it is exposed to high-energy photons from the UV light. This was done on a MJB4 manual mask aligner (SUSS MicroTec). The chrome mask is placed above the silicon wafer covered with resin and the silicon wafer is exposed to UV light only through the pattern on the mask. The exposure duration was 4.5 s with the vacuum contact between the mask and the wafer. The exposure energy is close to 20  $\text{mJ cm}^{-2} \text{s}^{-1}$ .
5. Directly after exposure we did a post exposure bake on hotplate for 3 min at 95°C.
6. To remove the parts of the resin that did not solidify during the exposure to UV light, we used SU-8 developer solvent. The wafer was strongly agitated for 2 minutes on a shaker. At the end it was washed with isopropyl alcohol and carefully air dried with compressed air.
7. The same procedure was repeated for the 25  $\mu\text{m}$  thick nutrient channel layer. We used SU-8 2035 which was spun in two steps, 500 rpm for 10 s and 6000 rpm for 30 s. Then it was soft baked for 3 min at 65°C and 6 min at 95°C. The mask was properly aligned with the existing cell chamber pattern and the wafer was exposed to UV light for 10 s. Post exposure bake was done for 1 min at 65°C and 6 min at 95°C. After a 5-minute-long development, the wafer was rinsed and dried.
8. Then we checked the dimensions of the master wafer using Dektak 150 surface profiler (Veeco).
9. Finally, we treated the master wafer with (3-Mercaptopropyl)trimethoxysilane 95% (Sigma) for 1 hour in vapour phase.

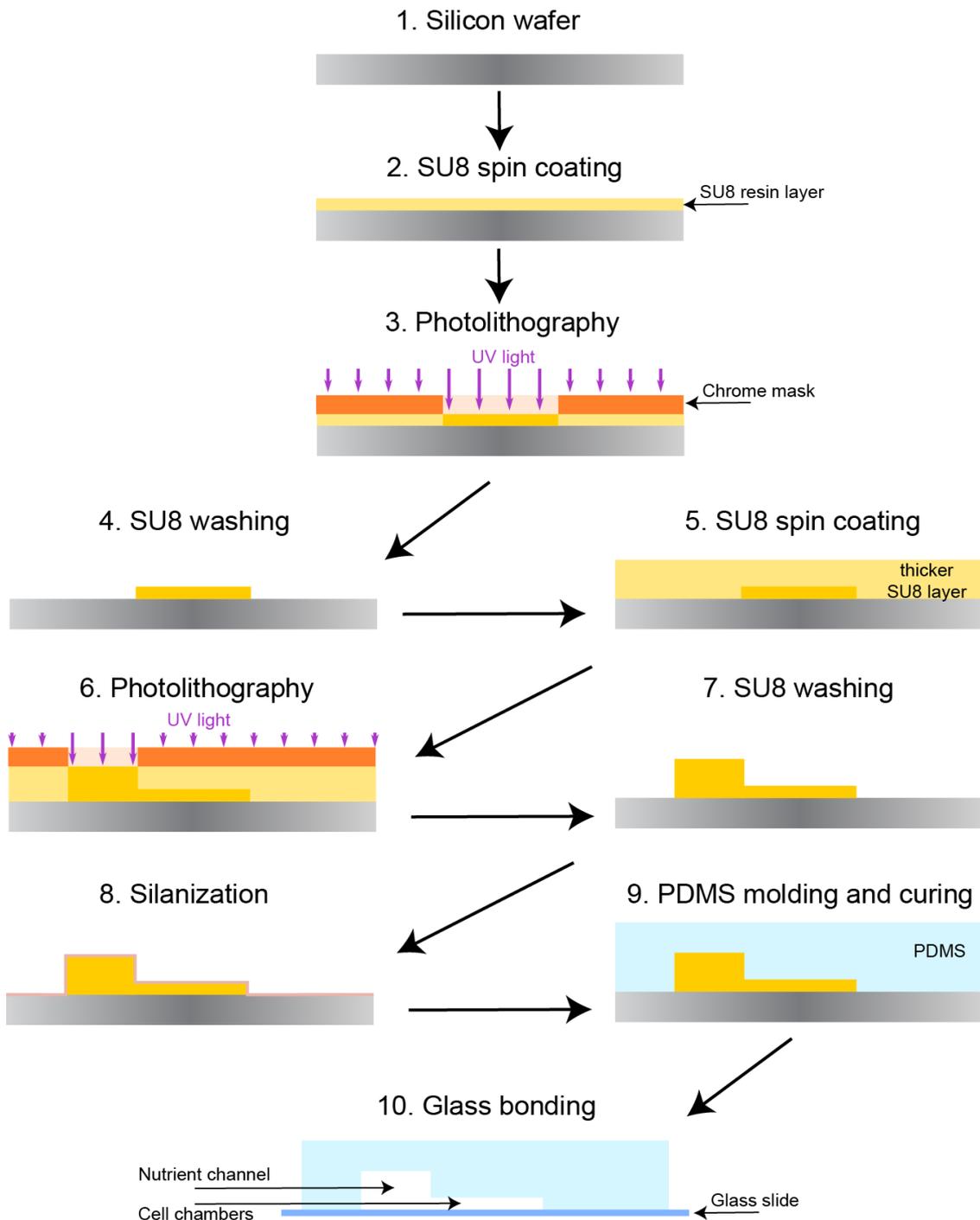


Figure 96 Microfluidic device fabrication. Epoxy-based resin is spread on a silicon wafer and illuminated with the UV light through a custom designed chrome mask. Soluble part of the resin is washed off and a new cycle of resin deposition can be made depending on the number of layers of the microfluidic device. The master wafer is silanized and used as a re-usable negative mold to produce microfluidic chips. Polydimethylsiloxane (PDMS) is then poured on the master wafer and cured until it is polymerized. The PDMS chip is bonded with a glass coverslip by plasma bonding. The final microfluidic chip is used to grow cells and deliver nutrients. The thinner parts are the cell chambers, and the thicker part is the main nutrient channel. Figure adapted from [179].

Now that we have the desired pattern on the master wafer, we can use it as a negative for replica moulding of the “Yeast Machine” chip with the help of polydimethylsiloxane (PDMS). PDMS is a transparent silicon that is a viscous fluid when it is monomeric and it is elastic once it homopolymerizes in a reaction catalysed by a curing agent. The “Yeast Machine” was made by casting a degassed 10:1 mix of PDMS and curing agent (Sylgard 184 kit; Dow Corning) on the master wafer, followed by at least 2 hours of curing at 65°C. The chip was then gently cut and peeled off the master wafer, the fluid/cell entry/exit ports were punched out and the scotch tape was used to remove all the particles from the PDMS chip surface. The chip and a glass coverslip (24 x 50 mm #1; Menzel-Gläser) were treated with O<sub>2</sub> plasma for 1 minute in a plasma cleaner (Harrick Plasma), bonded together to make a strong irreversible bond and put at 65°C for 10 minutes for final bonding. Before cell loading the chip was coated with 1% Pluronic F-127 (Sigma) for 30 minutes.

Cells were first grown overnight in 5 mL of Synthetic Complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids (BD Difco) + 0.79 g/L complete supplement mixture (CSM; MP Biomedicals)) containing 2% glucose (VWR) in a shaking incubator at 30°C, then they were diluted 50 times in 50 mL of SC + 2% glucose and grown for 5-6 hours in a shaking incubator at 30°C to an OD<sub>600</sub> of 0.2-0.4. Cells were centrifuged, loaded into the flow channel with a pipette and the chip was centrifuged for 2 minutes at 1000 rpm (Laurell WS-650 spin coater) in order for cells to enter the dead-end cell chambers. The chip was mounted on the microscope and the input/output tubing was connected to it. Liquid media was strongly flown through the flow channel to remove the remaining cells and the flow rate was set to 5µL/min. Pressure based microfluidic flow control system was used to push liquid media through the flow channel (MFCS; Fluigent) coupled with a flow rate platform (Fluigent) and a flow rate control module (Fluigent) which measured the flow rate and kept it constant by adjusting the pressure through a feedback loop (Figure 97). The output was kept under the constant pressure of 100 mbar on top of the atmospheric pressure to minimize the bubble formation inside the flow channel while the pressure at the input determined the flow rate.

For time-lapse imaging, we used an inverted fluorescence motorized microscope (IX81, Olympus) equipped with an EMCCD camera (Evolve 512, Photometrics) and X-Cite exacte fluorescence light source (Lumen Dynamics). Optical filters from Chroma Technology Corporation ET-EGFP (U-N49002; Ex 470/40 Di495 Em 525/50) and ET-DsRed (U-N49005; Ex 545/30 Di570 Em620/60) were used for observing GFP and RFP fluorescence. Cells were observed by an Olympus 10x (Plan 10x / 0.25 NA), 60x (PlanApo N 60x / 1.42 NA Oil) and

100x (UPlanFL N 100x / 1.3 NA Oil) objectives. The open source microscopy software  $\mu$ Manager [75] was used to control all of the above components. The temperature inside the microscope incubation chamber which contained the media and the cells was held constantly at 30°C (Life Imaging Services). Fluorescence intensity was set to 10% of the maximum output, fluorescence exposure was set to 1000 ms and camera gain was set at maximum. The time interval between each exposition cycle was 6 minutes.

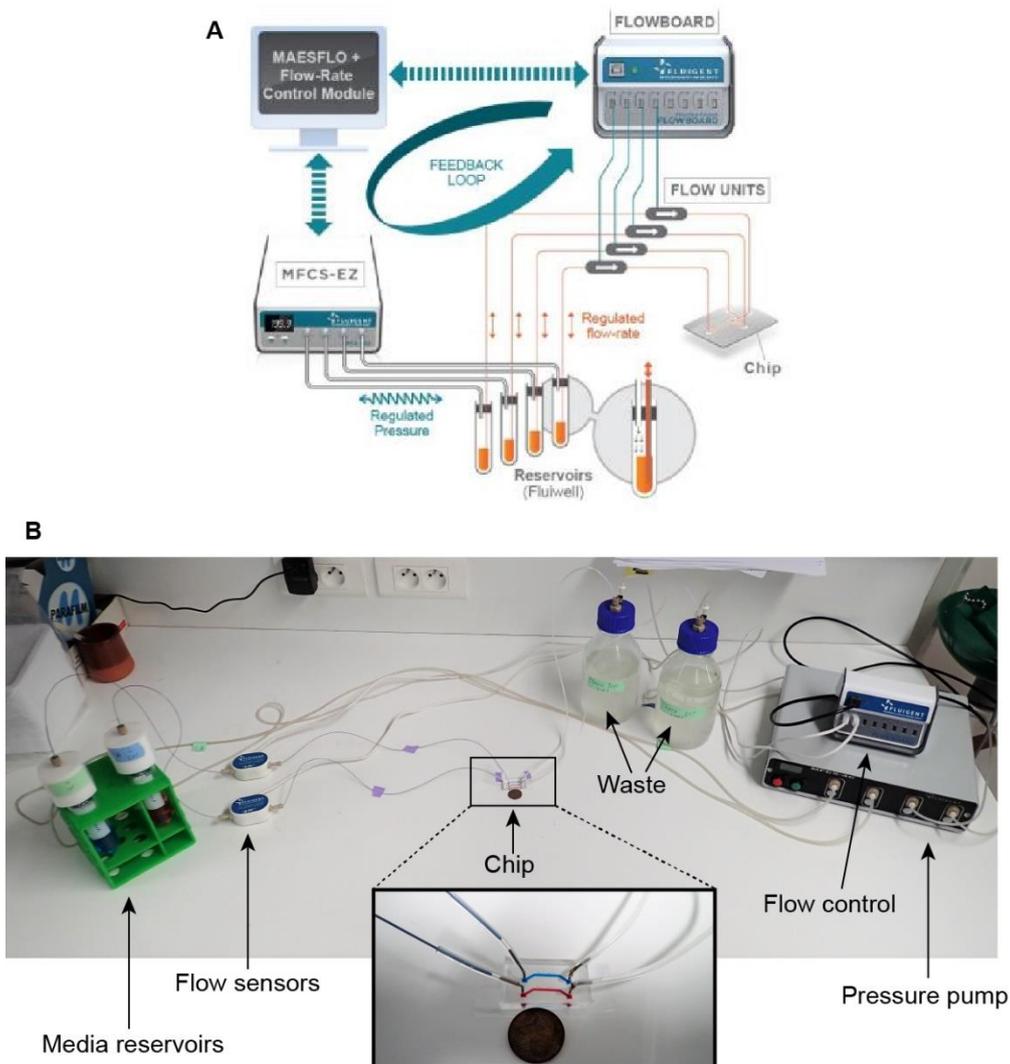


Figure 97 The experimental setup for the “Yeast Machine”. (A) Pressure based microfluidic flow control system from Fluigent. Media reservoirs are pressurized with the help of MFCS pressure pump. This pressure makes the nutrients flow through flow sensors into the chip and then to waste. Flow sensors and pressure pump are connected through the flow rate control module which keeps a constant flow through the system. Figure from [www.fluigent.com](http://www.fluigent.com). (B) The experimental setup in real life detached from a microscope.

## The steep path to the best chip design

The development of the proper technique and the architecture of the “Yeast Machine” might seem simple and easy but it took eighteen chip designs and almost two years to get to the stage where experiments and the experimental protocol was robust enough to produce good results.

At the beginning we wanted to mimic the growth of cylindrical yeast that I mentioned before [149], so nutrients had to be delivered at the bottom of the colony and cells would grow upwards and then be washed away by the media that did not contain glucose. This way cells that are dividing would stay at the bottom and would not be washed away by the flow. It was also important to separate the channel with glucose and the cell chamber so a filter was added between them so that cells cannot invade the channel with glucose and that they have a leverage to grow unidirectionally upwards. At first, we experimented with very wide chambers, 1000  $\mu\text{m}$  wide and 300  $\mu\text{m}$  long (Figure 98 A). In this setup cells did not move linearly, they were flowing left and right, sometimes vigorously washed away by the flow at the top. We tried to add U-shaped traps to limit cell movement and to keep a certain portion of cells at a constant position (and, we then thought, in constant environment), but that device did not work out too well either (Figure 98 B). Then we tried to design narrower cell chambers of different shapes (funnels, straight lines) to constrict movement and let the cells grow linearly in one direction (Figure 98 C, D, E). This device was promising until we discovered a crucial glitch. There was a crossflow between the two channels through the cell chamber, meaning that nutrients did not enter the cell chamber by diffusion but rather sometimes during experiment there would be a flow through the chamber from one channel to the other. This happened when pressures on both sides became uneven, like when air bubbles would pass through one channel or when pressure fluctuations in our nutrient delivery system were too big to pass a certain tolerable limit calculated in the paper by Levchenko’s group [165]. Their device is, so far, the only one that we know of to have a design with two nutrient channels and open cell chamber between them. They used it to create pheromone gradients and because they were able to control the pressure on both sides very precisely they did not have a crossflow between two channels. This is why we switched to a dead-end design with only one nutrient channel and it took some time to find right parameters of length, width, and height as well as solving cell loading, media procurement, bubbles removal, stick-slip, chip surface passivation, and media leakage due to poor bonding between the chip and the glass coverslip (Figure 98 F).

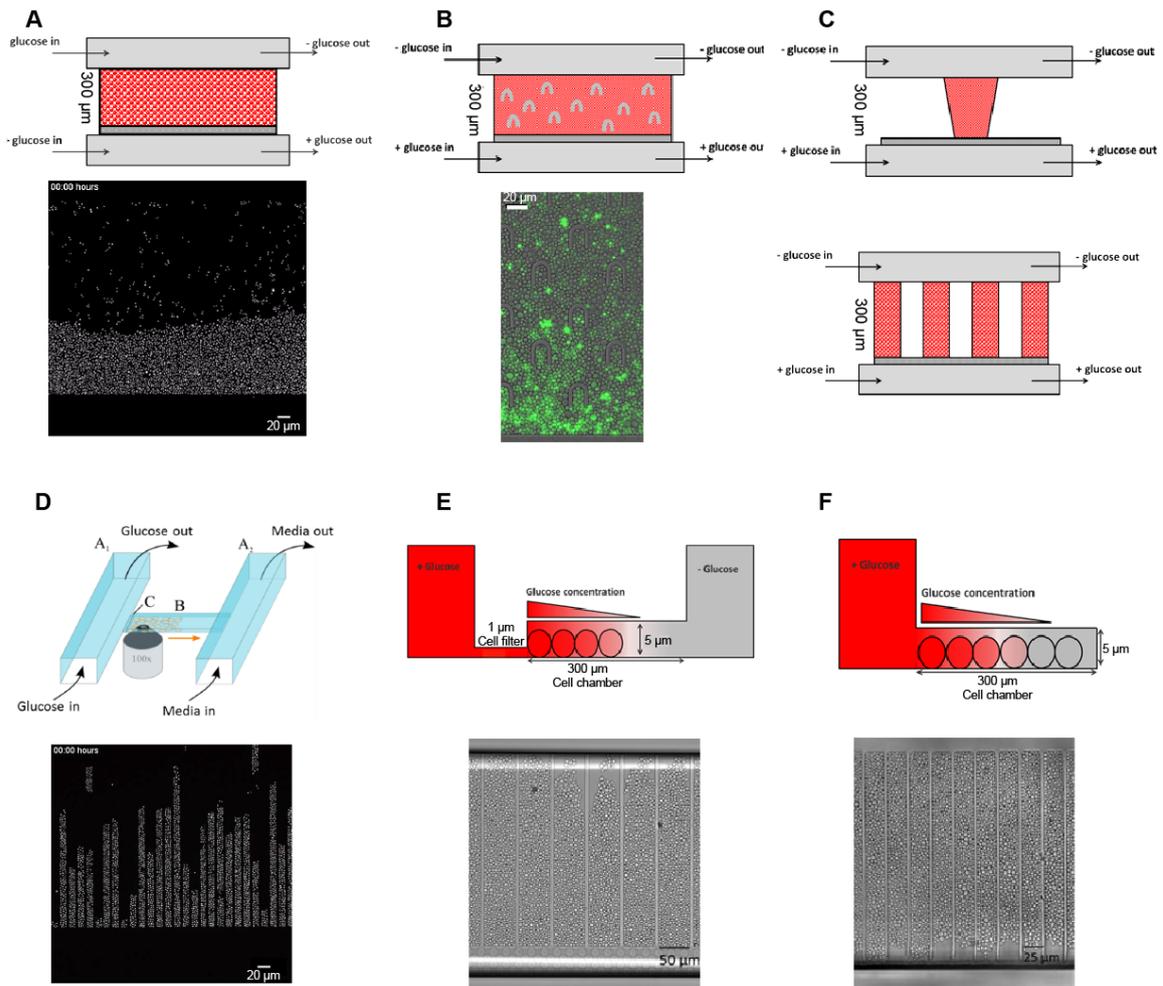


Figure 98 Many iterations of the “Yeast Machine” design. (A) 300 μm long and 1000 μm wide device with two nutrient channels for large populations of yeast. The idea was to provide glucose on the bottom and no glucose on top. Bottom channel and cell chamber were separated by a 1 μm thin filter so cells would grow only upwards. (B) Same as the previous design but with U-shaped traps that would keep a certain portion of cells at one position instead of pushing them through the colony. (C) Narrower cell chambers shaped as funnels or lines. (D, E) Design with 300 μm long and 10-50 μm wide cell chambers. Thin cell filter separates cell chambers and glucose channel so that cells do not invade it and grow only unidirectionally away from glucose. Figure adapted from [13]. (F) Short “Yeast Machine”. Only 300 μm long.

In the words of Samuel Beckett *“Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.”*, the conception of the “Yeast Machine” went through ups and downs, trials and errors, failings and successes, but with each step it got better. Here I list some of the issues I encountered during the optimization of our microfluidic system which should be understood as tips for better and efficient design of similar microfluidic devices in the future:

*Cell filter collapsing.* The most time consuming technical aspect that I was trying to solve was the collapsing of the cell filter after the plasma bonding on the cover slip. I tried to change different aspects in the wafer and chip manufacturing process like cell filter size, cell filter architecture, PDMS stiffness, plasma cleaning time, plasma bonding time, the force applied to the chip and cover slip, post bonding baking, and cover slip type. None of the changes affected the collapsing significantly until the resolution of the produced patterns in the cell filter was improved in the microfabrication process. The combination of low UV light exposure time and 1.35  $\mu\text{m}$  high cell filter, together with the 5:1 ratio of monomer:curing agent, 50 s plasma clean, Duran glass coverslip, >10 min @ 65°C after PDMS bondage to the glass coverslip finally resulted in no cell filter collapse.

*Bubbles.* The experiments are very sensitive to bubbles. They can block the channels and stop the flow of media, lower the channel diameter which changes the flow rate, disturb the cells and cause the flow through the cell chambers while passing through the media channels by making the pressure differences between the two channels. This problem was quite severe for some time and it made many experiments unusable. I tried many different techniques to prevent the bubble formation but the best and most stable technique was to slightly pressurize the media outlet too, in addition to the pressurized inlet.

*Cells bursting through cell filter.* At the beginning the cells were bursting through the cell filter quite often. After some adjustments to the filter architecture and the strength of the binding to the cover glass the cells stopped bursting through the pillars in almost all of the experiments.

*Stick-slip.* Since the PDMS chip is elastic, during growth cells can locally accumulate and then at one point relax and spread around. This is called stick-slip effect. In some of the experiments the stick-slip effect was very obvious. To reduce it we had to find the right height of the cell chamber and a good surface passivation method.

*Loading the cells.* It is hard to load the cells in the “Yeast Machine” chip without centrifuging them. For this purpose, we designed a previously described 3D printed device to hold the chip in the spin coater.

*Pump.* The peristaltic pump was used at the beginning but it produced too much instability in the system because of the pulsing flow so I switched to the Fluigent pressure based pump to get highly stable flows. With the upgrade that

added the flow rate sensors and a controller that can hold the flow at a constant rate the media procurement stopped to be a problem.

*Crossflow between the media channels.* As mentioned before, crossflow between the media channels was unsolvable in our system so we had to switch to a significantly different design.

*Surface passivation.* I started to use Pluronic F-127 instead of BSA for surface passivation. It is a cheaper and more stable molecule than BSA. I wet the chip with 1% Pluronic F-127 and wait for 30 minutes. Then I load the cells. The cells go nicely into the chamber when centrifuged and there is no fluorescent background. This helped to reduce the stick-slip too.

Looking back, it was a hard road to the most optimal “Yeast Machine” design. But all the experience proved to be well invested once a fully functional device with characteristics that I previously described started to produce good and reliable results.



## Yeast strains

Transporter and sensor deletion experiments were done using haploid *S. cerevisiae* strains derived from the CEN.PK.2-1C background MAT $\alpha$  leu2-3,112 ura3-52 trp1-289 his3- $\Delta$ 1 MAL2-8 SUC2. All the other experiments were done using haploid *S. cerevisiae* strains derived from the S288c background - BY4741: MAT $\alpha$  his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0. We would like to thank Sébastien Léon for kindly letting us use his strains. <http://www.ijm.fr/en/research/research-groups/leon/>

Name	Background	Genotype	Source
yPH001	BY4741	MAT $\alpha$ his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0	Léon's Lab
yPH152	BY4741	HXT1-GFP :HisMX <sub>fds</sub>	Léon's Lab
yPH155	BY4741	HXT7-GFP::HphNT	Léon's Lab
yPH179	BY4741	HXT2-GFP::HisMX	Yeast GFP collection
yPH180	BY4741	HXT3-GFP::HisMX	Yeast GFP collection
yPH182	BY4741	GLK1-GFP::HisMX	Yeast GFP collection
yPH183	BY4741	MIG1-GFP::HisMX	Yeast GFP collection
yPH188	BY4741	PDC1-GFP::HisMX	Yeast GFP collection
yPH189	BY4741	HXK2-GFP::HisMX	Yeast GFP collection
yPH190	BY4741	HXK1-GFP::HisMX	Yeast GFP collection
yPH191	BY4741	SDH2-GFP::HisMX	Yeast GFP collection
yPH192	BY4741	HXT4-GFP::HisMX	Léon's Lab
yPH193	BY4741	HXT5-GFP::HisMX	Léon's Lab
yPH236	BY4741	HXT6-GFP::Hyg	Léon's Lab
yPH_220	CEN.PK.2-1C	MAT $\alpha$ leu2-3,112 ura3-52 trp1-289 his3- $\Delta$ 1 MAL2-8 SUC2 HXT7-yEGFP::kan	Hersen's Lab
yPH_221	CEN.PK.2-1C	EBY.VW4000 HXT7-yEGFP::kan	Hersen's Lab
yPH_223	CEN.PK.2-1C	EBY.VW5000 HXT7-yEGFP::kan	Hersen's Lab



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## Side projects

During my PhD I took part in two additional projects. I was a mentor of the Paris Bettencourt iGEM team in 2013 and 2014 (and a student member in 2012). iGEM is a major student competition in synthetic biology. In 2013, we worked on a project called “Fight Tuberculosis with Modern Weapons” which goal was to diagnose and eliminate *Mycobacterium tuberculosis*. The details of this project can be found here [http://2013.igem.org/Team:Paris Bettencourt](http://2013.igem.org/Team:Paris_Bettencourt) and I attached two papers that we published as a result of this project. This project also won the Grand Prize in the iGEM competition. In 2014, we worked on a project “The smell of us” which goals was to engineer the human microbiome to eliminate unpleasant body odour. The details of the project can be found here [http://2014.igem.org/Team:Paris Bettencourt](http://2014.igem.org/Team:Paris_Bettencourt)

I also worked on a project with Adrien Hallou, my lab colleague, who studied the pattern formation in *Dictyostelium discoideum* aggregates in confined microenvironments. I helped with some biological aspects of the project, mostly molecular cloning, and the manuscript for it is currently in preparation, and it should be published next year.

## Silencing of Antibiotic Resistance in *E. coli* with Engineered Phage Bearing Small Regulatory RNAs

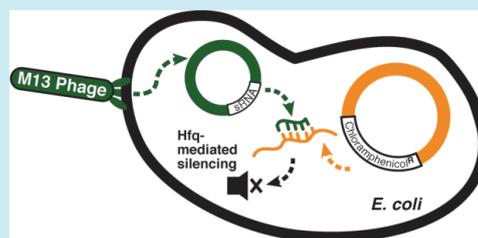
Vincent K. Libis,<sup>†,§</sup> Aude G. Bernheim,<sup>†,§</sup> Clovis Basier,<sup>†</sup> Sebastián Jaramillo-Riveri,<sup>†</sup> Matthew Deyell,<sup>†</sup> Idonnya Aghoghogbe,<sup>†</sup> Iva Atanaskovic,<sup>†</sup> Amel Camélia Bencherif,<sup>†</sup> Marguerite Benony,<sup>†</sup> Nicolas Koutsoubelis,<sup>†</sup> Anne C. Löchner,<sup>†</sup> Zoran S. Marinkovic,<sup>†</sup> Sarah Zahra,<sup>†</sup> Yonatan Zegman,<sup>†</sup> Ariel B. Lindner,<sup>\*,†,‡</sup> and Edwin H. Wintermute<sup>\*,†,‡</sup>

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### Supporting Information

**ABSTRACT:** In response to emergent antibiotic resistance, new strategies are needed to enhance the effectiveness of existing antibiotics. Here, we describe a phagemid-delivered, RNA-mediated system capable of directly knocking down antibiotic resistance phenotypes. Small regulatory RNAs (sRNAs) were designed to specifically inhibit translation of chloramphenicol acetyltransferase and kanamycin phosphotransferase. Nonlytic phagemids coding for sRNA expression were able to infect and restore chloramphenicol and kanamycin sensitivity to populations of otherwise resistant *E. coli*. This modular system could easily be extended to other bacteria with resistance profiles that depend on specific transcripts.



The emergence of resistance to conventional antibiotics has motivated interest in techniques to extend or complement their effectiveness.<sup>1</sup> Bacteriophage act to kill bacteria through mechanisms distinct from those of small-molecule antibiotics. Therefore, bacteriophage may serve as complementary therapeutics or bacterial control agents in some contexts.

As with classical antibiotics, the direct application of bacteriophage to cause cell death rapidly selects for resistant strains.<sup>2</sup> Combination therapies that employ multiple phage or drugs can slow the evolution of resistance. Previous work has shown that bacteriophage and antibiotics can act synergistically, with modified bacteriophage altering host physiology to increase drug sensitivity.<sup>3</sup>

In this work, we developed an M13 bacteriophage to knock-down the expression of two genes responsible for antibiotic resistance in *E. coli*: chloramphenicol acetyltransferase and kanamycin phosphotransferase (Figure 1). We used rationally designed, sequence-specific sRNAs to target and silence the transcript for each gene directly.<sup>4</sup> Because such sRNAs can be designed to complement any mRNA, the approach could extend to any antibiotic resistance bestowed by specific transcripts.

The system is RNA-mediated and does not require additional protein expression. Further, the construct is delivered by a nonlytic phagemid system and targets only antibiotic-resistance transcripts. By limiting the disruption of host physiology we have sought to minimize the fitness burden imposed by the construct. In this way, the evolutionary pressures favoring

phage-resistance are minimized until a lethal dose of antibiotic can be applied to a formerly resistant population.

## RESULTS AND DISCUSSION

Following the design of Na et al.,<sup>4</sup> we created two sRNA expression constructs targeting chloramphenicol acetyltransferase and kanamycin phosphotransferase (Figure 2). These constructs were incorporated into an M13 phagemid, which was then assembled and released by *E. coli* with the help of an M13 phagemid helper plasmid. Because phagemids do not code for viral structural proteins, we reasoned that a phagemid-based delivery would minimize the physiological disruption and fitness cost of our construct to the infected host.<sup>5</sup>

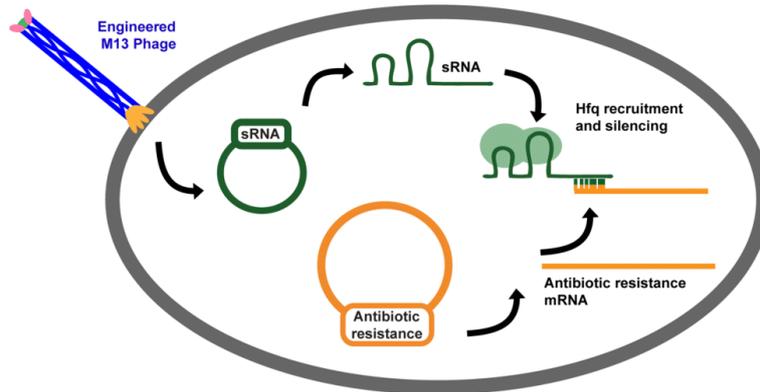
As described in the Methods, populations of chloramphenicol or kanamycin resistant *E. coli* were infected with phagemid bearing the relevant sRNA silencing construct. Antibiotic sensitivity was assayed by plating on chloramphenicol or kanamycin at a range of concentrations.

Our construct restored substantial antibiotic sensitivity to otherwise resistant populations (Figure 3). At high chloramphenicol concentrations (500  $\mu\text{g}/\text{mL}$ ), we observed >99% killing for infected populations, but no significant killing in control populations (Figure 3a). Lower chloramphenicol concentrations killed less efficiently, but the effect of the

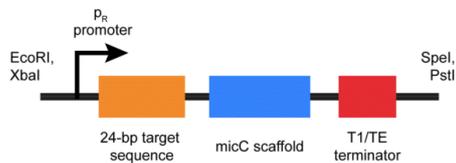
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**Figure 1.** Scheme for antibiotic silencing with phagemid-delivered sRNA. M13 bacteriophage capsules infect the *E. coli* host and deliver a modified nonreplicating phagemid bearing the sRNA expression construct. A 24 bp complementary sequence in the sRNA targets the antibiotic resistance mRNA, while a MicC scaffold sequence recruits the Hfq protein to promote hybridization and degradation.<sup>4</sup>



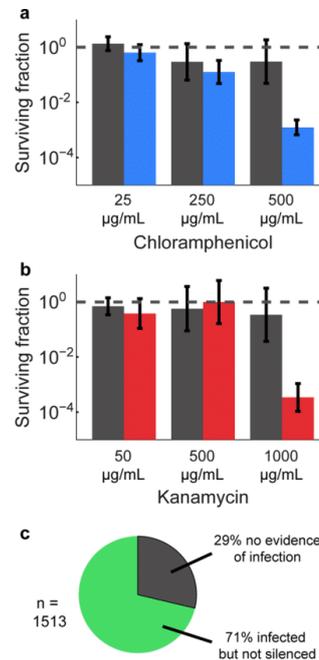
**Figure 2.** Structure of the sRNA module for the silencing of chloramphenicol acetyltransferase. The entire 419 bp construct was synthesized and cloned into a phagemid vector with BioBrick-compatible restriction sites. The 24 bp target sequence is complementary to the +1 to +24 sequence of the target gene. The modular design is easily changed to target other resistance-associated mRNAs.

sRNA was still evident at 250  $\mu\text{g}/\text{mL}$ . Similar results were obtained for kanamycin (Figure 3b).

We observed significant variability among biological replicates in our plating assay, particularly at intermediate antibiotic concentrations (Supporting Information Table S1). Plating efficiency may have been affected by the stress of high-antibiotic concentrations, even in genetically resistant cells. Other sources of variation may include variable titers or the accumulation of mutations in phage collected from overnight cultures. In the case of the Kanamycin-silencing construct, phagemids were produced using the M13K07 phagemid-helper plasmid. This plasmid itself contains a target for the expressed sRNA. Although the plasmid was maintained at kanamycin concentrations below the levels at which we observed reduced resistance, this interaction may have contributed to the accumulation of mutations.

Because our observed killing efficiencies were less than 100%, we next sought to investigate the ways in which bacteria may escape the killing effect. A cell may escape killing in our system either because it does not become infected with the phagemid or because the phagemid does not effectively silence resistance. Because our phagemid also codes for GFP expression, we were able to estimate the infection rates of the surviving cells.

We scored GFP fluorescence, indicating phagemid infection, in 71% of surviving colonies (Figure 3C). Thus, in the majority of cases, cell survival is due to a failure of the sRNA to adequately silence gene expression. This may be due



**Figure 3.** Engineered phagemids restore antibiotic sensitivity to a genetically resistant population. Bacterial populations carrying plasmid-borne resistance cassettes for chloramphenicol (a) and kanamycin (b) were infected with sRNA-expressing phagemids. In the infected populations, significant killing was observed especially at high antibiotic concentrations. In control populations with no sRNA-silencing construct, no significant killing was observed. Dotted lines indicate 100% survival. Error bars represent 95% confidence intervals. (c) Failure modes for sRNA-mediated silencing. Phagemid-encoded GFP was used to score surviving colonies for the presence of the phagemid. The expression of GFP in 71% of surviving colonies (68–74 at 95% confidence) indicates that phagemid infection was successful but sRNA-mediated silencing did not act to restore antibiotic sensitivity.

insufficient production of the sRNA. The pACYCDuet-1 and pCOLADuet-1 vectors that were used to convey antibiotic resistance are estimated by the manufacturer (Novagen) to be present at copy numbers of 10–12 and 20–40, respectively. The sRNA-targeted transcripts may be in excess of sRNA production. Alternately, the sRNA-mRNA complex may be too slow to form or too weakly bound to completely silence transcription. Finally, mutations in the antibiotic resistance genes could disrupt sRNA targeting.

Future work could address in greater detail the mechanism by which resistant *E. coli* escape silencing. Some routes of escape are easily addressed with simple and modular sRNA design. For example, point mutations in the antibiotic resistance gene could be easily matched with a redesigned sRNA. Other escape mechanisms, such as overexpression of the target mRNA or resistance to phage infection, would be more problematic. In particular, mutations in the *pili* are known to confer resistance to M13 phage.<sup>2</sup>

The counter-selection of our construct in a bacterial population will be in proportion to the fitness burden it imposes on the host. We have taken steps to reduce the burden of our construct by avoiding protein expression, by minimizing the size of the coding sequence and by targeting only antibiotic resistance transcripts. The targeted genes are nonfunctional in any antibiotic-free medium and costly to translate.<sup>6</sup> A low-burden construct should be more easily maintained in bacterial population prior to the administration of lethal antibiotic doses. However, further population genetic modeling will be required to minimize counterselection and account for the specific dynamics of a particular host–phage system.

Phage therapy and phage-based biocontrol predate the development of conventional antibiotics.<sup>7</sup> Currently, broad-spectrum small molecule drugs remain the antibacterial weapon of choice in most cases, despite recent challenges in the form of evolved resistance. Integral to the general success of antibiotics was the application of synthetic chemistry to improve the stability, specificity, and activity of naturally occurring antibiotic compounds. Increasingly, Synthetic Biology provides the tools to enhance wild phage isolates.<sup>8</sup> Combination therapies featuring semisynthetic antibiotics and designer phage may synergistically increase the value of both approaches.

## METHODS

### Design of an sRNA to Target Antibiotic Resistance.

The method of Na et al.<sup>4</sup> was used to design sRNA transcripts to silence chloramphenicol acetyltransferase and kanamycin phosphotransferase. A 24 bp sequence complementary to the +1 to +24 area of each target gene was inserted within the MicC scaffold sequence, which promotes RNA hybridization through recruitment of the Hfq protein. The  $P_R$  promoter and T1/TE terminators were included to control the transcription of the regulatory RNA. With the addition of BioBrick-compatible restriction cloning sites, the complete 419 bp module was synthesized by IDT. Restriction cloning was used to integrate the sRNA-expression module into Litmus28i\_J23115-B0032-GFP, an M13-derived phagemid carrying GFP.<sup>9,10</sup>

**Construction of Phage and Antibiotic Resistant Strains.** Phage-producing cells were created by cotransforming *E. coli* MG1655 with a phagemid and phagemid-helper plasmid M13K07 (NEB). After overnight culture of the phage-producers in selective LB, phage were isolated by centrifugation and filtration at 0.2  $\mu\text{m}$ .

Antibiotic resistant strains were created by transforming *E. coli* MG1655 with pACYCDuet-1 or pCOLADuet-1 plasmids (Novagen) to convey resistance to chloramphenicol or kanamycin, respectively. An F plasmid was also introduced to both strains by conjugation, as F pilus expression is required for sensitivity to M13.

**Antibiotic Sensitivity Assay.** Resistant *E. coli* were grown to log phase ( $\text{OD}_{600} = 0.7$ ) in selective LB. One milliliter of culture was mixed with 100  $\mu\text{L}$  of harvested phage or plain LB as a negative control. Phage infection was allowed to proceed for 30 min at 37 °C with agitation. Serial dilutions were then plated on selective LB agar with defined antibiotic concentrations. Sensitivity to a given antibiotic was assayed by comparing CFU counts at a specific antibiotic concentration to counts on antibiotic-free LB agar.

**Phage Infection Assay.** Following infection and plating, the number of phagemid-carrying *E. coli* was estimated by scoring GFP fluorescence of single colonies by eye using an Illumatool model LT-9900 (Lighttools Research) with appropriate filters for GFP.

**Statistical Analysis.** For the antibiotic sensitivity assay, the survival fraction was calculated as a CFU count on antibiotic plates compared to a matched antibiotic-free control. Confidence intervals were estimated using the standard error of the mean for the log-transformed survival fraction. For the phage infection assay, confidence was estimated as a binomial proportion confidence interval.

## ASSOCIATED CONTENT

### Supporting Information

Exact DNA sequences for the sRNA-expressing phagemids created for this study; raw survival data for the antibiotic sensitivity assay depicted in Figure 3a, b. This material is available free of charge via the Internet at <http://pubs.acs.org>. Further discussion of this project and its context within the larger work of the 2013 Paris Bettencourt iGEM team is available via our team Web site at [http://2013.igem.org/Team:Paris\\_Bettencourt](http://2013.igem.org/Team:Paris_Bettencourt).

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### Author Contributions

<sup>§</sup>V.K.L. and A.G.B. contributed equally to this work. V.K.L., A.G.B., and E.H.W. designed the experiments. A.G.B., V.K.L., and C.B. conducted the experiments. All authors contributed to the analysis and interpretation of results. V.K.L., A.G.B., and E.H.W. prepared the manuscript.

### Notes

The authors declare no competing financial interest.

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## In Situ Characterization of Mycobacterial Growth Inhibition by Lytic Enzymes Expressed in Vectorized *E. coli*

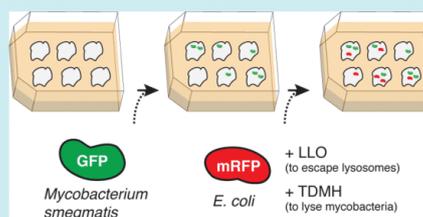
Iva Atanaskovic,<sup>†,§</sup> Amel Camélia Bencherif,<sup>†,§</sup> Matthew Deyell,<sup>†</sup> Sebastián Jaramillo-Riveri,<sup>†</sup> Marguerite Benony,<sup>†</sup> Aude G. Bernheim,<sup>†</sup> Vincent K. Libis,<sup>†</sup> Nicolas Koutsoubelis,<sup>†</sup> Yonatan Zegman,<sup>†</sup> Anne C. Löchner,<sup>†</sup> Clovis Basier,<sup>†</sup> Idonnya Aghoghogbe,<sup>†</sup> Zoran S. Marinkovic,<sup>†</sup> Sarah Zahra,<sup>†</sup> Matthias Toulouze,<sup>†</sup> Ariel B. Lindner,<sup>\*,†,‡</sup> and Edwin H. Wintermute<sup>\*,†,‡</sup>

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**ABSTRACT:** The emergence of extremely drug resistant *Mycobacterium tuberculosis* necessitates new strategies to combat the pathogen. Engineered bacteria may serve as vectors to deliver proteins to human cells, including mycobacteria-infected macrophages. In this work, we target *Mycobacterium smegmatis*, a nonpathogenic tuberculosis model, with *E. coli* modified to express trehalose dimycolate hydrolase (TDMH), a membrane-lysing serine esterase. We show that TDMH-expressing *E. coli* are capable of lysing mycobacteria *in vitro* and at low pH. Vectorized *E. coli* producing TDMH were found suppress the proliferation of mycobacteria in infected macrophages.

**KEYWORDS:** mycobacteria, TDMH, LLO, protein vectors, macrophages, tuberculosis



*M. tuberculosis* infects macrophages within the lungs where it is difficult to reach with conventional antibiotics. This problem is compounded by the highly impermeable mycobacterial membrane, which also resists the normal process of phagocytosis. The recently discovered enzyme TDMH, a serine esterase, can enzymatically lyse the mycobacterial cell envelope by digesting outer-membrane trehalose mycolates.<sup>1</sup>

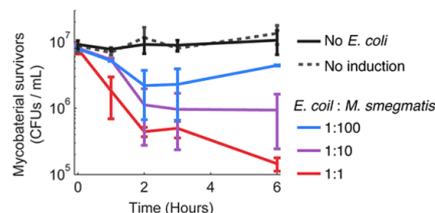
Previous work has shown that engineered *E. coli* can serve as a vector to deliver active proteins to the macrophage cytosol.<sup>2</sup> Briefly, lysteriolysin O (LLO) forms large pores in the phagosomal membrane that allow the passage of proteins to the cytosol. *E. coli* that express LLO are phagocytosed and lysed, causing the release of both LLO porin and their protein payload.

In this work, we explore the feasibility of using *E. coli* as a vector to deliver TDMH to mycobacteria-infected macrophages. We show that individual *E. coli* are capable of expressing sufficient TDMH to lyse more than 10 mycobacteria in mixed cultures and that TDMH is active at low pH matching the phagosomal environment. Further, we show that engineered *E. coli* can colocalize with mycobacteria and can halt mycobacterial spread in cultured macrophages.

### RESULTS AND DISCUSSION

Following Yang<sup>1</sup> and Higgins,<sup>2</sup> we created an *E. coli* strain capable of inducible expression of both TDMH and LLO. *M. smegmatis* served as a nonvirulent model for *M. tuberculosis* because it is known to exhibit comparable membrane structure and macrophage infection dynamics.<sup>3</sup>

We first sought to verify that TDMH-expressing *E. coli* were able to kill mycobacteria in coculture (Figure 1). We achieved nearly 99% killing of mycobacteria after 6 h when *E. coli* and



**Figure 1.** TDMH-expressing *E. coli* kill mycobacteria in mixed cultures. *M. smegmatis* and *E. coli* populations were mixed in LB at defined ratios and TDMH expression was induced with IPTG. Viability was assayed at defined time points by serial dilution and plating. Nalidixic acid plates were used to select and count only *M. smegmatis* from the mixed population. Nearly 99% killing of *M. smegmatis* was recorded after 6 h when the strains were mixed in equal proportion (red line). Significant killing was also apparent for *E. coli*: *M. smegmatis* ratios of 1:10 (purple line) and 1:100 (blue line). No change in viability was recorded when *M. smegmatis* were cultured alone (black line) or mixed 1:1 with uninduced *E. coli* (gray dotted line). Error bars represent 95% confidence intervals calculated from at least 3 biological replicates.

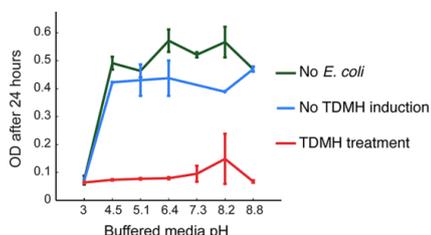
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mycobacteria were mixed in equal proportion and TDMH expression was induced. Uninduced *E. coli* produced no significant killing. Varying the ratio of *E. coli* to *M. smegmatis* indicated that effective killing required approximately one fully induced *E. coli* for every 10 mycobacteria.

The phagosomal pH is significantly more acidic than batch culture media, varying from 4.8 to 5.4 under most conditions.<sup>4</sup> We next tested if TDMH-mediated killing could function at low pH (Figure 2). *M. smegmatis* growth was measured with and



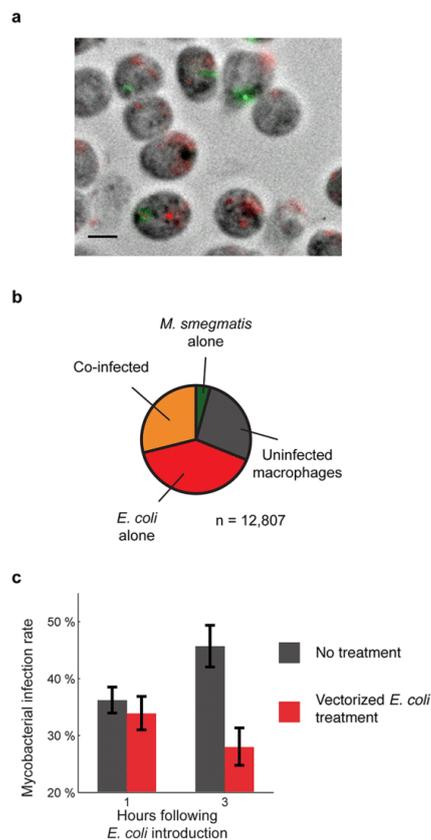
**Figure 2.** TDMH functions to inhibit mycobacterial growth at low pH. Media supernatant from induced and uninduced TDMH-producing *E. coli* was applied to *M. smegmatis* cultures growing in LB buffered to the indicated pH. Mycobacterial growth was assayed by a plate reader after 24 h. At pH 3, no growth was detected for any treatment. Mycobacteria treated with TDMH-induced *E. coli* supernatant (red line) showed no growth after 24 h at any pH tested. Mycobacteria treated with only LB (green line) or supernatant from uninduced *E. coli* (blue line) grew to saturation. The effect of TDMH on mycobacterial growth was independent of pH.

without supernatant collected from TDMH-expressing *E. coli* in media buffered to pH values ranging from 3 to 8.8. At very low pH values, *M. smegmatis* was unable to grow regardless of TDMH treatment. At pH levels from 4.5–8.8, *M. smegmatis* was able to grow and TDMH was functional as a growth inhibitor. This suggests that TDMH activity is retained at phagosomal pH.

We next evaluated the capacity of vectorized *E. coli* to colocalize with mycobacteria within infected macrophages (Figure 3a, b). We exposed a population of macrophages first to GFP-expressing *M. smegmatis*, then to RFP-expressing *E. coli*. Using fluorescence microscopy, we visually scored the bacteria taken up by each macrophage. We found that 26% of macrophages contained both *E. coli* and *M. smegmatis* while only 4% contained *M. smegmatis* alone ( $n = 12\,807$ ). The high rate of coinfection indicates that macrophages containing *M. smegmatis* remain active and are able to take up vectorized *E. coli* in most cases.

Finally, we applied *E. coli* expressing both TDMH and LLO to treat macrophages preinfected with mycobacteria (Figure 3c). Addition of vectorized *E. coli* significantly reduced mycobacterial infection rates in a population of macrophages after 3 h, as determined by fluorescence microscopy. A control population of macrophages, infected with only GFP-expressing *M. smegmatis*, showed a significant increase in mycobacterial infection rate over the same time period. This suggests that vectorized *E. coli* are able to slow or reverse the spread of mycobacteria within macrophages.

The antimycobacterial activity of vectorized *E. coli* might be improved by optimizing the activity, expression and mechanism of the relevant enzymes. A variety of bacteriolytic enzymes are in common laboratory use and could be adapted for this



**Figure 3.** Vectorized *E. coli* localize to mycobacteria-infected macrophages and slow the spread of mycobacteria in a macrophage population. (a) *E. coli* expressing LLO and TDMH (labeled with RFP) were introduced to macrophages along with *M. smegmatis* (labeled with GFP). After 1 h, colocalization was scored by fluorescence microscopy at 40 $\times$  magnification. Scale bar is 5  $\mu$ m. (b) 26% of imaged macrophages contained *E. coli* and *M. smegmatis*, compared to 4% containing *M. smegmatis* alone. This indicates that *E. coli* can enter mycobacteria-infected macrophages at high frequency. (c) Mycobacterial localization in macrophages was scored 1 h following coinfection with *E. coli* and again after 3 h. Mycobacterial infection rates declined in a macrophage population treated with vectorized *E. coli* (red bars). In control macrophages treated with mycobacteria alone, mycobacterial infection rate increased significantly after 3 h (gray bars). This may indicate the activity of TDMH in lysing mycobacteria and inhibiting growth. Error bars represent 95% confidence intervals of at least 3 biological replicates.

purpose. Cell-envelope degrading enzymes like TDMH may be particularly valuable if they can act synergistically with existing antimycobacterial drugs like isoniazid or ethambutol that target cell-envelope biogenesis. Potential synergy may also be found with the oxidative, acidic, and enzymatic stresses that weaken bacterial membranes within the phagosome.

While synthetic *E. coli* have previously been proposed as antimicrobials, a common challenge is directing their activity in a complex microenvironment.<sup>5</sup> In the case of intraphagosomal mycobacteria, we have shown that it is possible to make use of

the existing immune response to target a bacterial payload. Vectorized *E. coli*, by delivering functional protein systems to the intracellular macrophage, can enhance its natural microbicidal activity.

## METHODS

**Strains and Fluorescent Labeling.** The BL21(DE3) strain of *E. coli* was used for all experiments. RFP labeling of *E. coli* was achieved with a pCOLA plasmid carrying BBa\_J23102 from the Registry of Standard Biological Parts. *Mycobacterium smegmatis* MC2 155 labeled with pMyco-*gfp*<sup>6</sup> was a gift from Stéphane Canaan (CNRS). J774 macrophages were obtained from Nicolas Hegerle and Nicole Guiso (Institut Pasteur).

**TDMH and LLO Expression.** TDMH expression was achieved with the IPTG-inducible plasmid pET21b carrying Msmeg\_1529 (TDMH), provided by Dr. Anil Ojha (University of Pittsburgh). Constitutive LLO expression was achieved with the plasmid pACYC184 encoding *hly* (LLO), provided by Dr. Daniel A Portnoy (UC Berkeley).

**Culture Conditions and Killing Assay.** *M. smegmatis* and *E. coli* were grown separately in LB media to saturation in biological triplicates. The strains were diluted by a factor of 100 and mixed 1:1 in a 10 mL volume, and full induction of TDMH expression was achieved with 1 mM IPTG. At defined time periods, viable *E. coli* and *M. smegmatis* were assayed by serial dilution and selective plating. Nalidixic acid (3 µg/mL) was used to select and count only *M. smegmatis* colony-forming units in the mixed cultures. Kanamycin (100 µg/mL) similarly selected for only *E. coli* carrying pCOLA-RFP.

**pH Dependence of TDMH Activity.** *M. smegmatis* was resuspended at an initial OD of 0.05 in LB buffered with phosphate to a range of pH values. Filter-sterilized supernatant was collected from saturated *E. coli* cultures induced for TDMH expression, or uninduced cultures as a control. Supernatant (20 µL) was added to 200 µL of *M. smegmatis* culture and growth was followed by OD on a plate reader after 24 h. In all cases, measurements were performed in biological triplicate.

**Macrophage Experiments and Microscopy.** Macrophages were grown in 24 well plates in RPMI 1640 media supplemented with 1% glutamine, 10% of fetal bovine serum, 1% HEPES, and 1% sodium pyruvate. Experiments began when the cells approached 90% confluence.

*E. coli* and *M. smegmatis* were grown to saturation, washed 3 times with RPMI media, and resuspended at an OD of 0.1. 100 µL of each bacterial strain was added to 1 mL of macrophage culture. Cell mixtures were incubated for 1 h without agitation (37 °C, 5% CO<sub>2</sub>), then washed 10 times with PBS and resuspended in fresh RPMI.

For the coinfection experiments (Figure 3a, b), the presence of *E. coli* or *M. smegmatis* was scored visually using a Nikon Eclipse Ti-E inverted fluorescent microscope. We collected 119 images from 6 separate wells representing 3 biological replicates. In total, 12 807 macrophages were scored.

For the *in situ* growth-inhibition experiments (Figure 3c), GFP-expressing *M. smegmatis* were grown to saturation, washed, and introduced to macrophages as above. Macrophages were either untreated or preinfected with vectorized *E. coli* expressing TDMH, LLO and RFP. The percentage of macrophages infected with *M. smegmatis* was scored visually after 1 and 3 h by fluorescence microscopy. At least 500 cells from at least 6 images were scored for each data point.

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### Author Contributions

§I.A. and C.B. contributed equally to this work. C.B., I.A., and E.H.W. designed the experiments. C.B., I.A., and M.D. conducted the experiments. All authors contributed to the analysis and interpretation of results. I.A., C.B., and E.H.W. prepared the manuscript.

### Notes

The authors declare no competing financial interest.

Further discussion of this project and its context within the larger work of the 2013 Paris Bettencourt iGEM team is available via our team Web site at [http://2013.igem.org/Team:Paris\\_Bettencourt](http://2013.igem.org/Team:Paris_Bettencourt).

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# Additional figures

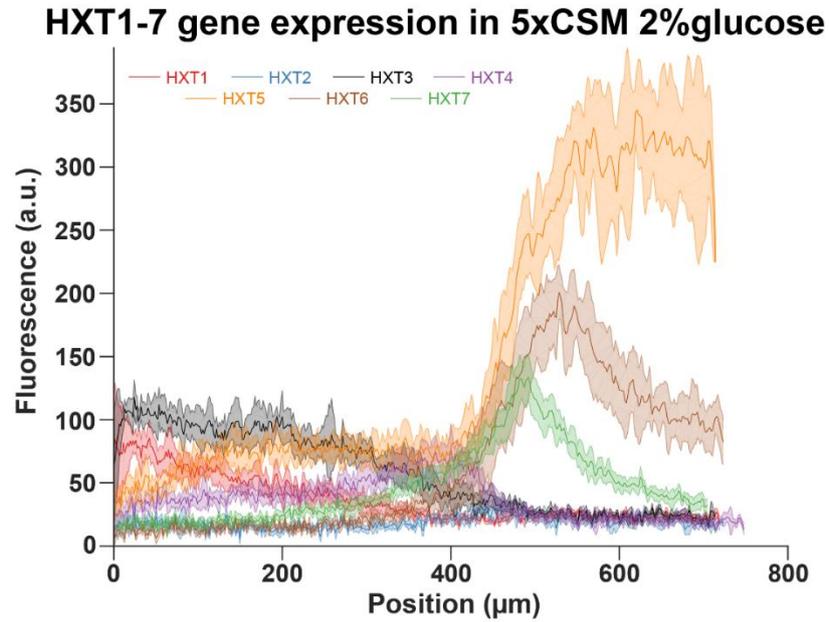


Figure 99 Gene expression of HXT1-7 genes in 2% glucose concentration.

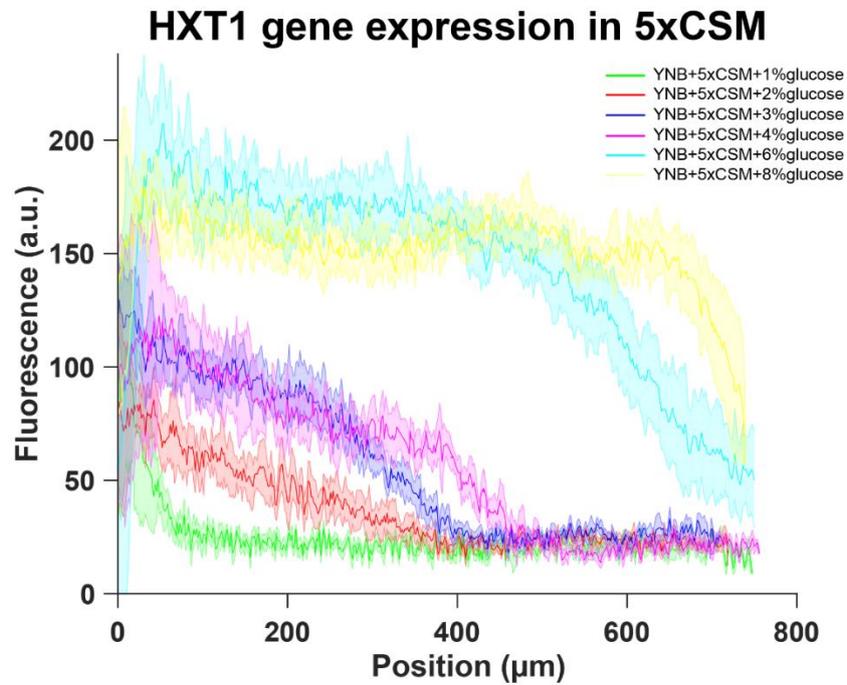


Figure 100 Gene expression of glucose transporter HXT1 in different glucose concentrations.

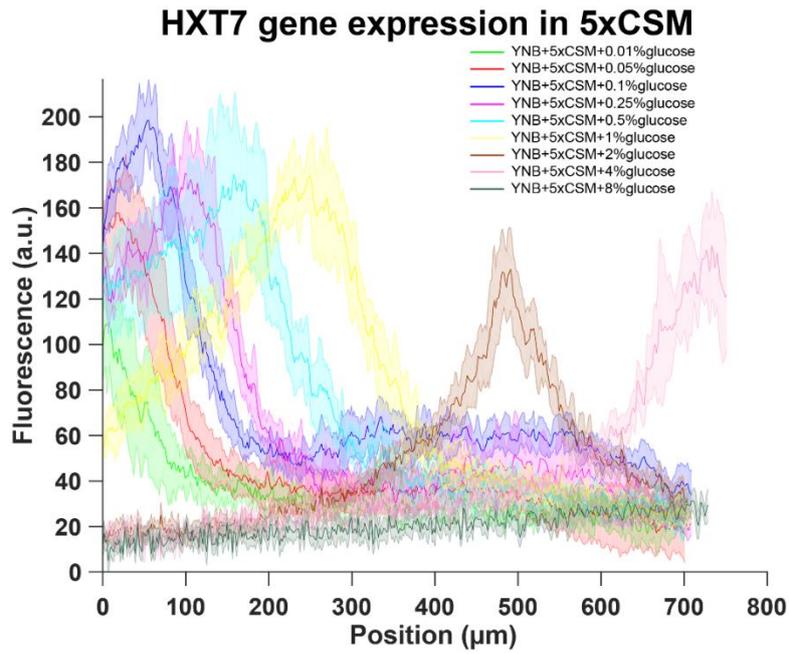


Figure 101 Gene expression of glucose transporter HXT7 in different glucose concentrations.

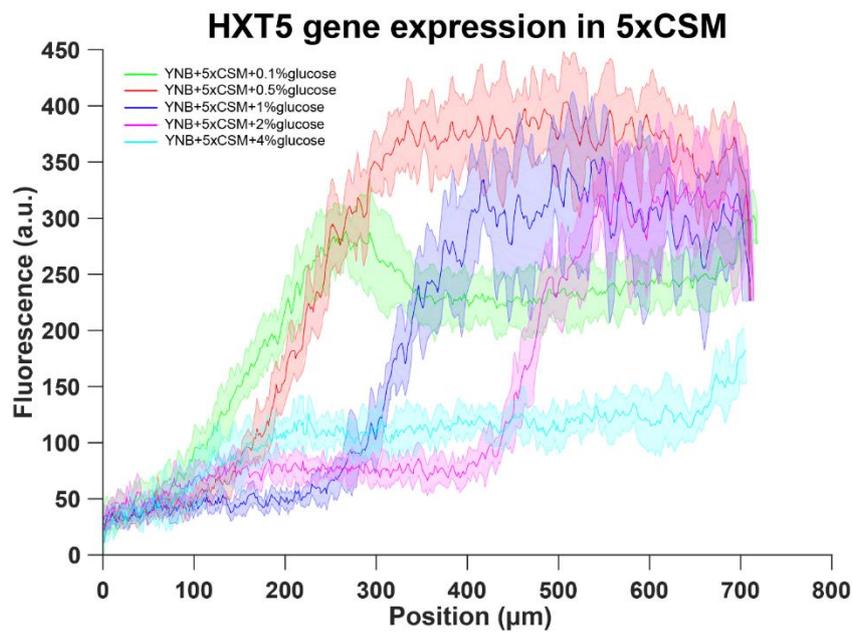


Figure 102 Gene expression of glucose transporter HXT5 in different glucose concentrations.

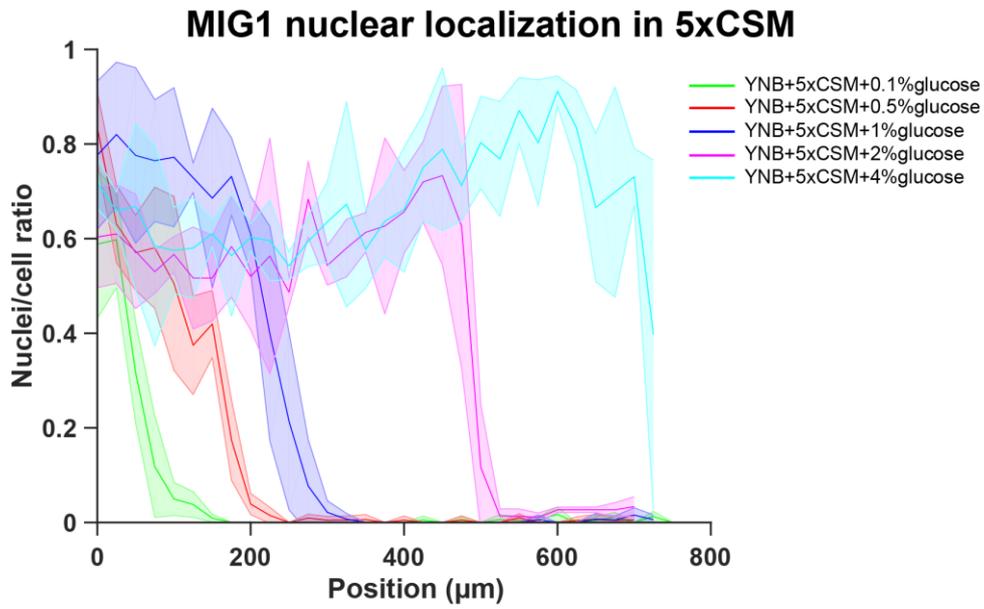


Figure 103 Gene expression of transcription factor MIG1 in different glucose concentrations.

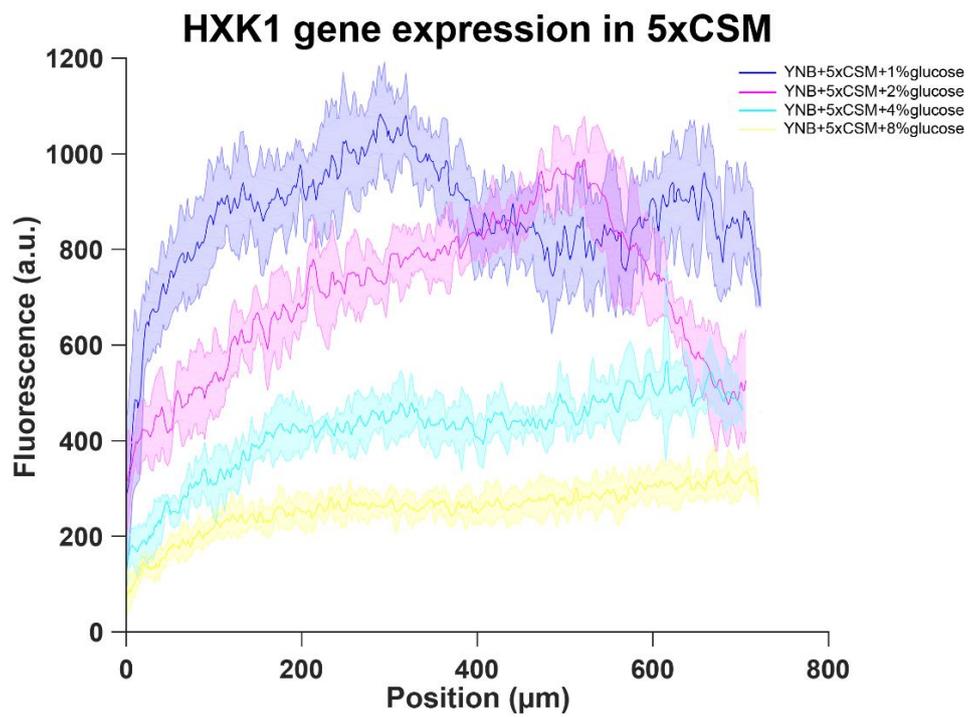


Figure 104 Gene expression of hexokinase 1 (HXK1) in different glucose concentrations.

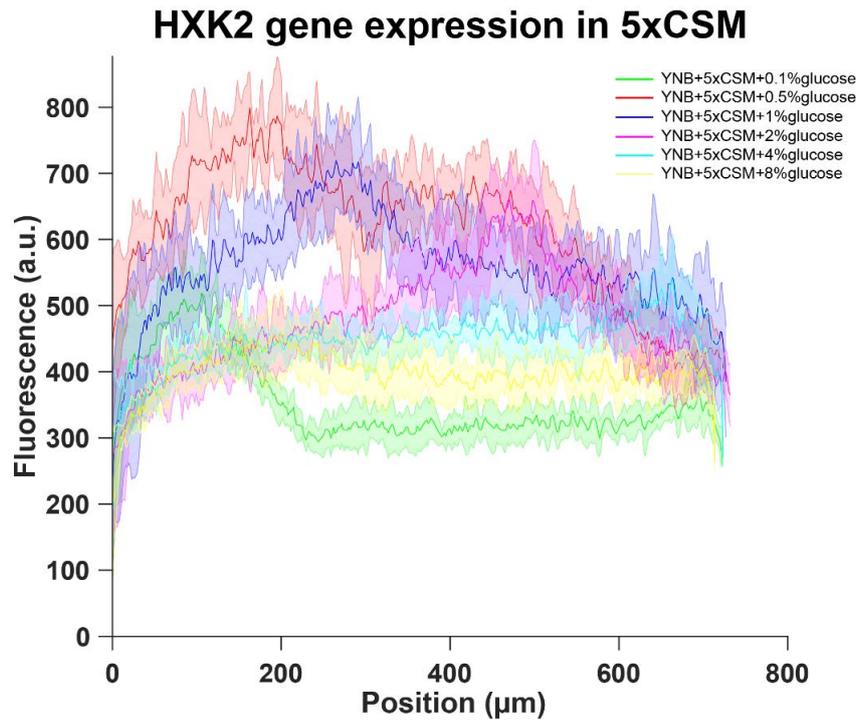


Figure 105 Gene expression of hexokinase 2 (HXK2) in different glucose concentrations.

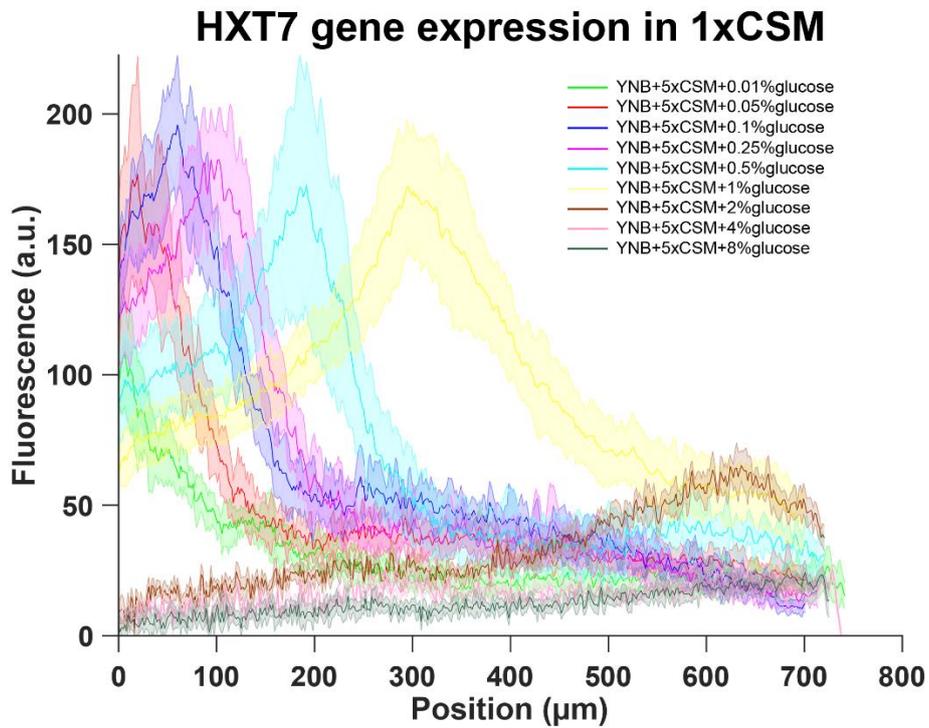


Figure 106 Gene expression of glucose transporter HXT7 in different glucose concentrations.

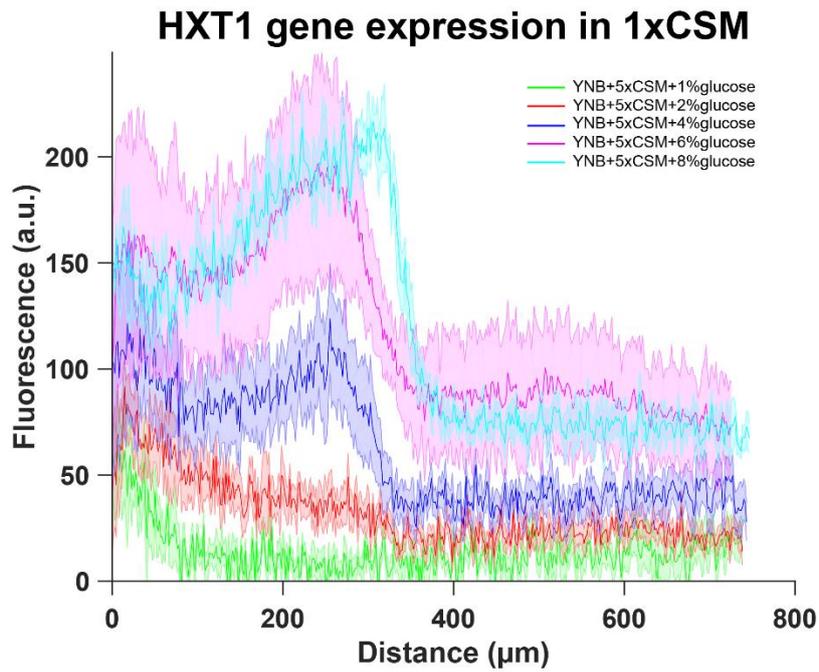


Figure 107 Gene expression of glucose transporter HXT1 in different glucose concentrations.

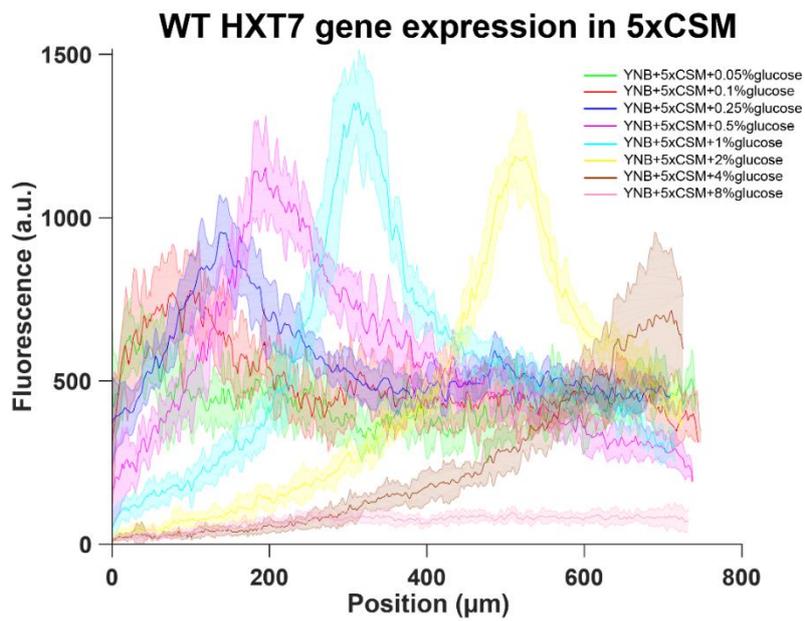


Figure 108 Gene expression of glucose transporter HXT7 in different glucose concentrations.

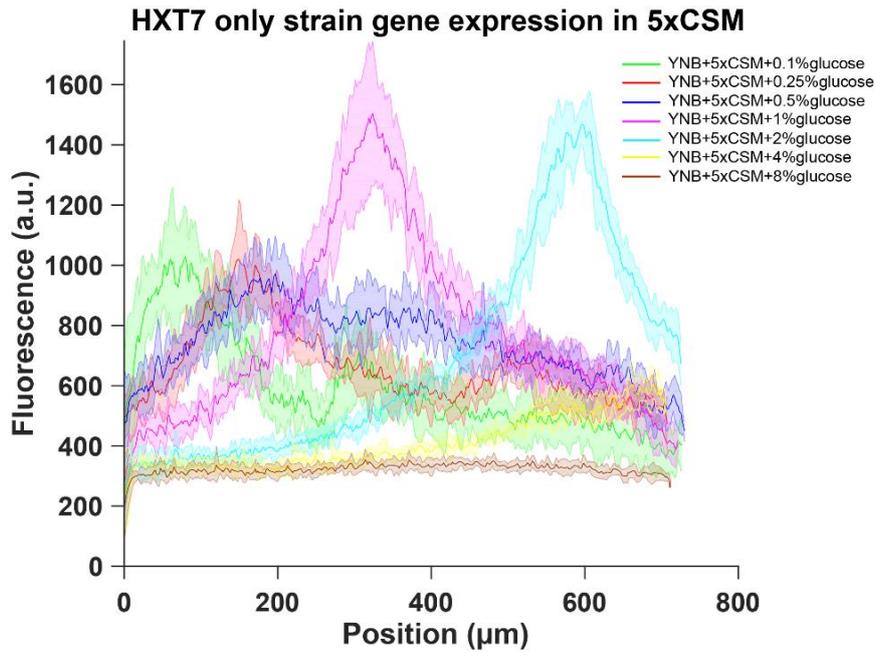


Figure 109 Gene expression of glucose transporter HXT7 in HXT7 only strain in different glucose concentrations.

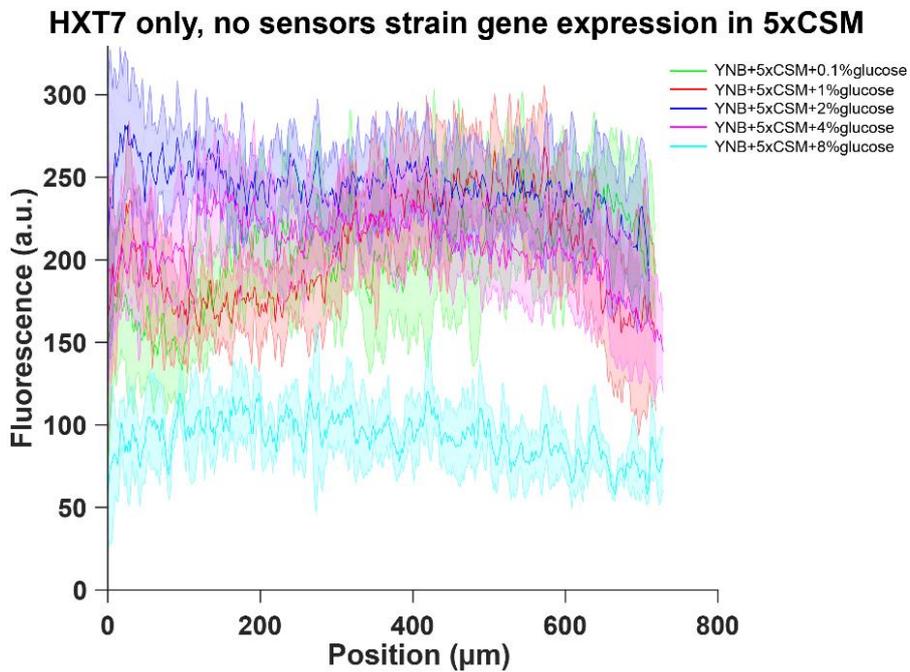


Figure 110 Gene expression of glucose transporter HXT7 in sensorless HXT7 only strain in different glucose concentrations.



