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Assembly and Quantification of Co-Cultures Combining Heterotrophic Yeast with Phototrophic Sugar-Secreting Cyanobacteria

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Citation

Hasenklever, D., Pohlentz, J.C., Berwanger, T., Kokarakis, E.J., Hassan, T., Schipper, K., Matuszyńska, A., Axmann, I.M., Ducat, D.C. Assembly and Quantification of Co-Cultures Combining Heterotrophic Yeast with Phototrophic Sugar-Secreting Cyanobacteria. *J. Vis. Exp.* (214), e67311, doi:10.3791/67311 (2024).

Date Published

December 27, 2024

DOI

10.3791/67311

URL

jove.com/video/67311

Abstract

With the increasing demand for sustainable biotechnologies, mixed consortia containing a phototrophic microbe and heterotrophic partner species are being explored as a method for solar-driven bioproduction. One approach involves the use of CO_2-fixing cyanobacteria that secrete organic carbon to support the metabolism of a co-cultivated heterotroph, which in turn transforms the carbon into higher-value goods or services. In this protocol, a technical description to assist the experimentalist in the establishment of a co-culture combining a sucrose-secreting cyanobacterial strain with a fungal partner(s), as represented by model yeast species, is provided. The protocol describes the key prerequisites for co-culture establishment: Defining the media composition, monitoring the growth characteristics of individual partners, and the analysis of mixed cultures with multiple species combined in the same growth vessel. Basic laboratory techniques for co-culture monitoring, including microscopy, cell counter, and single-cell flow cytometry, are summarized, and examples of nonproprietary software to use for data analysis of raw flow cytometry standard (FCS) files in line with FAIR (Findable, Accessible, Interoperable, Reusable) principles are provided. Finally, commentary on the bottlenecks and pitfalls frequently encountered when attempting to establish a co-culture with sugar-secreting cyanobacteria and a novel heterotrophic partner is included. This protocol provides a resource for researchers attempting to establish a new pair of co-cultured microbes that includes a cyanobacterium and a heterotrophic microbe.

Introduction

With the rapid expansion of genomic tools and DNA technologies in recent years, bioengineering efforts are increasingly able to consider mixed communities of microbes as viable for bioproduction strategies rather than solely focusing on axenic cultures. Microbial consortia hold multiple potential advantages relative to single-species cultures, including specialization and division of labor, adaptability and robustness, and efficiency of substrate utilization[1]. However, the predictable engineering of multi-species consortia is complicated by uncertainties caused by higher-order behaviors that emerge from inter-species interactions[2]. Cross-species signaling and metabolite exchange are at the heart of the principle of division of labor but also lead to unexpected synergies and antagonisms between participants of the consortia[3]. Considerable development in the field is necessary if the full potential of mixed microbial consortia can be realized, including the use of flexible 2- and 3-partner co-culture platforms, which can be used to better characterize and understand microbial interactivity from the "bottom up."

A few dominant types of co-culture platforms are currently in use within the field, including complimentary auxotrophic partners and microbes that secrete metabolites that are generally beneficial to a broad range of microbial species. In the latter category, cyanobacteria have been engineered to become enhanced primary producers through the introduction of pathways that lead to the secretion of easily metabolized carbohydrates and have now been explored in a variety of rationally designed consortia. Briefly, in such engineered microbial communities, the cyanobacterial partner is capable of utilizing light and CO_2 as the primary inputs, and through the process of oxygenic photosynthesis, these strains can secrete central carbon sugars as a public good. One class of such engineered cyanobacterial strains are those that have been engineered to secrete the disaccharide sucrose[4]. Such strains have likely enjoyed their success because sucrose is a metabolite that is close to central carbon metabolism for many species and is also frequently hyper-accumulated as a so-called "compatible solute" to adapt to a variety of environmental abiotic stresses[5]. A minimal number of genetic interventions can lead to efficient sucrose secretion in a range of cyanobacterial model organisms[4].

Sucrose-secreting cyanobacteria are a useful platform for the investigation of rationally designed microbial consortia because a wide range of heterotrophic species can metabolize sucrose as their dominant source of carbon and energy. Indeed, utilizing a few model cyanobacteria with sucrose-secreting capabilities, many laboratories have rationally designed mixed species co-cultures and consortia that contain one or more heterotrophic microbes and which are ultimately supported by the primary inputs of light and CO_2 without supplementation of organic carbon feedstocks[4]. The heterotrophic strains may simply subsist on the cyanobacterial-derived carbohydrates, or they may be utilized to convert the sucrose feedstock into higher-value bioproducts (e.g., fuels, polymers, pigments, etc.). In addition to being a potential strategy for sustainable bioproduction, such simplistic co-cultures may also be useful as a platform for the investigation of emergent interactions between unrelated microbial species.

This video article focuses on methodologies and prerequisites for utilizing sugar-producing cyanobacteria as a flexible platform for the design of simple microbial consortia that can

be stable by supplementing only light and inorganic carbon inputs. While the setup and monitoring of cultures containing single established microorganisms are mostly straightforward and can easily be achieved using optical density (OD) or backscatter methodology, this is not feasible once two or more organisms are combined in one vessel. The major reason is that these methods do not distinguish between the different microorganisms, hence, they only provide an overall picture of the culture and do not resolve growth of the individual organisms. Moreover, cyanobacteria have a wide absorption spectrum in the 400-750 nm range, so to measure the OD_{600} of a heterotroph would lead to false results due to phycocyanin (that absorbs in 620 nm). Therefore, specific protocols for the setup of cyanobacteria-heterotroph mixed communities within the laboratory as well as useful generic protocols for the analysis of the performance of these consortia over time, are provided. While the protocols focus on a specific pairing of a model, sucrose-secreting cyanobacterial species with one or more model heterotrophic microbe, the intent of this work is to provide a resource for researchers who might wish to design new species pairings and to accelerate the optimization phase for the establishment of such cultures. Therefore, in addition to species-specific protocols, information and strategies that can be used to adapt and generalize these protocols for custom communities, as defined by the reader's needs, are included.

Because of the flexibility of the co-culture platform described herein, protocols for a number of different heterotrophic species that have been previously reported in co-cultivation with sugar-secreting cyanobacteria are described. For instance, the step-by-step protocol for a co-culture of *Synechococcus elongatus* PCC 7942 with the common laboratory yeast *Saccharomyces cerevisiae* is provided. Yet, the article also includes protocols that are appropriate for assaying the performance of co-cultures containing other model species, including the yeast form of *Ustilago maydis*.

The article focuses on a core set of protocols necessary to establish a cyanobacteria/heterotrophic co-culture and perform basic characterization of the performance of these mixed consortia over time. Specifically, single-cell flow cytometry and particle counting methods suitable to take an accurate census of different species, as well as microscopy approaches to evaluate cell morphology, are emphasized. These protocols are meant to serve as the basis for adaptation to the needs and equipment available. Importantly, technical notes and other considerations that are important for establishing and monitoring co-cultures within the laboratory are provided. Finally, examples of nonproprietary alternatives for data analyses of raw FCS[6] files using Python packages are included. In summary, the goal is to make cyanobacteria-based co-culture techniques more accessible to a wider scientific audience.

Protocol

NOTE: This protocol contains detailed instructions on how to set up and quantify co-cultures of sugar-secreting *S. elongatus* and heterotrophic model yeast species. In general, the protocol is applicable to any yeast species amenable to genetic manipulation.

1. Establishment of co-cultures combining phototrophic cyanobacteria and heterotrophic yeasts

1. Preparations for co-cultivation: Media and pre-cultivation

   NOTE: Prepare all media and stock solutions using ultrapure-filtered water. All glassware (measuring beakers, graduated cylinders, storage bottles, and cultivation flasks) should be cleaned and autoclaved.

Sterile media can be stored at room temperature (RT). A device compatible with the requirements of photoautotrophic cultivation needs to be available.

1. Prepare the co-culture medium at least 3-4 days in advance of the intended initiation of co-culture. Most co-culture media recipes are variations on the common BG-11 medium[7] utilized for routine cultivation of many cyanobacterial model species. This protocol uses the previously reported CoYBG-11 medium as an example ([8]; See **Table 1**). Autoclave (121 °C, 20 min) or filter sterilize (0.22 µm pore size) the prepared medium, as appropriate.
   NOTE: See the Discussion section for suggestions on adapting a medium appropriate for other species pairings.
2. Prepare molecular reagents: For induction of *cscB* expression and sucrose export[9,10,11], prepare a 1 M stock of isopropyl-β-thiogalactoside (IPTG).
   NOTE: Use of antibiotics during the co-culture is sub-optimal, but if all strains bear resistance cassettes, they can be used. In that case, prepare antibiotic stocks according to the needs of the employed strains.
3. Prepare a cyanobacterium preculture: At least 3 days in advance of initiation of the co-culture, transfer cells from a preculture of the sugar-secreting strain of cyanobacteria growing in BG-11 medium into a fresh baffled flask containing CoY^{BG-11} medium (**Table 1**).
   NOTE: Multiple species and strains of cyanobacteria have now been modified to secrete sugars. For a comprehensive review of strains available at the time of this writing, see[4]. This reference also lists heterotrophic species (see 1.1.4) for which cyanobacterial co-culture has been previously reported.
1. Measure the OD_{750} of the preculture. Calculate the required volume of the preculture to achieve a target OD_{750} of 0.3 (C_1) using the equation $V_1 = C_2 \times V_2/C_1$ where C_2 = target OD, V_2 = target volume, and V_1 = required volume of preculture. For example, when inoculating a 30 mL culture (V_2) the amount x of liquid culture to use is V_1 (mL) = (0.3 x 30)/(measured OD_{750}).
   NOTE: Optical density is an approximation of cell density and is dependent upon growth conditions and cell morphology; therefore, it is necessary for researchers to use methods to correlate optical density to absolute cell numbers (see sections 2.1, 2.2, and 2.3) for proper evaluation of co-culture performance. For cyanobacteria, light wavelengths within the photosynthetically active spectrum (i.e., 400-700 nm) should not be used for determining optical density because they are absorbed by chlorophyll and other pigments; therefore, far-red wavelengths are routinely used (e.g., 750 nm).
2. Incubate the diluted culture in an appropriate photoincubator. Typical incubation conditions for model cyanobacteria are as follows: 30 °C, 150 rpm (25 mm throw), 2% CO_2 headspace, ~200 µmol photons $m^{-2} \cdot s^{-1}$ LED lighting, 75% humidity.

NOTE: Supplementation with CO_2 is highly recommended to optimize the amount of released sucrose.

3. Repeat this dilution process each day for at least 3 days prior to setting up the co-culture so as to ensure that the cyanobacteria are in exponential growth. This will help improve consistency in co-culture performance between different-day experiments.

4. At least 2 days in advance of initiation of the co-culture, prepare a heterotroph preculture. As noted, a number of yeast species and substrains have previously been grown in co-culture with cyanobacteria[8,12,13].

1. Transfer cells in a 1:100 dilution (250 µL) from a liquid culture of the heterotrophic species growing in a rich cultivation broth (see **Table 1**) into 50 mL of CoY^{BG-11} medium supplemented with 20 g/L sucrose in a 250 mL baffled flask.

2. Incubate overnight at the same temperature as the cultivation conditions used for the cyanobacterial partner.

5. Check OD_{600} at least 24 h prior to the intended time of inoculation of co-culture to ensure that heterotrophic strain has grown sufficiently for experimental needs (see 1.2): dilute in fresh medium to maintain exponential growth if necessary.

2. Co-culture inoculation and maintenance

NOTE: See the Discussion section for considerations on the inoculation ratio between the photo- and heterotrophic microorganisms.

1. For inoculation of the co-culture, enrich both precultures by centrifugation.

1. Calculate the volume of concentrated cell suspension to achieve the desired starting density for both species using the standard $C_1 \times V_1 = C_2 \times V_2$ equation, where C_1 = OD of enriched culture. For instance, to make a 25 mL culture of *S. elongatus* with a starting OD_{750} of 0.5 (from a 1.5 OD axenic culture) and *S. cerevisiae* with a starting OD_{600} of 0.05 (from a 0.7 OD axenic culture), based on the above equation, 8.33 mL of *S. elongatus* starting culture, 1.79 mL of enriched heterotroph culture are needed, and 14.88 mL of CoY^{BG-11} medium.

2. Centrifuge the cultures using 13,000 x *g* for 10 min at RT. Under sterile conditions, decant and discard the supernatant.

NOTE: Less relative centrifugal force (RCF; xg) can be used to pellet *S. cerevisiae* as they are bigger cells (2x-3x times) in comparison to *S. elongatus,* but take care that the supernatant turns transparent. For the collection of the cyanobacterial biomass, less than 13,000 x *g* can also be used, but it would require a longer time for centrifugation (e.g., 4,000 x *g* can be used for 20 min).

3. Resuspend the cyanobacteria pellet in 25 mL of sterile CoY^{BG-11} medium. Use the same centrifugation conditions as previously, afterward, discard and decant supernatant.

4. Repeat this process twice for *S. elongatus* and 4 more times for *S. cerevisiae*.

NOTE: It is important to wash the pellets of cyanobacteria and the heterotroph (*S. cerevisiae*) to remove residual medium components. As *S. cerevisiae* had been growing in the presence of sucrose previously, the increased amount of washing steps will ensure that possible residual sugar will be removed.

5. Once the OD is verified, combine the volumes of *S. elongatus* and *S. cerevisiae* in a 250 mL baffled flask under sterile conditions and add CoY^{BG-11} to a final volume of 50 mL. Dilute from the previously prepared 1 M stock of IPTG to a final concentration of 1 mM (this is to induce the cyanobacteria to export sucrose to the media). For a 50 mL culture, use 50 µL from the 1 M IPTG stock.

   NOTE: Baffled flasks are usually better because they provide better aeration and mixing for cyanobacteria.

6. Place the flask from the previous step to 200 µmol photons·m^{-2}·s^{-1} LED lighting supplemented with 2% CO_2 at 30 °C with orbital shaking at 150 rpm (25 mm throw) and 75% humidity.

   NOTE: The above-mentioned conditions can vary and need to be optimized based on the experimental outcome.

7. Monitor the growth of the culture by sterile sampling (1 mL) every 12 h or 24 h (see section 2). Optionally use the samples to perform standard live cell microscopy and to determine colony-forming units (CFU^{14}) of yeast cells on a suitable medium to track cell morphology, and fitness and to monitor the amount of living heterotrophic cells as well as contamination.

   NOTE: CFUs are not recommended as a method to monitor the precise cell density of the cyanobacterial species, which can experience significant stress in the transition from cultivation in the liquid-to-solid medium. Therefore, agar plates with an appropriate rich medium for the growth of the yeast partner(s) species, such as yeast extract peptone dextrose (YEPD)[15], should be chosen.

Chemical compound	BG-11 (concentration mg/L)	CoY BG-11 (concentration mg/L)
$NaNO_3$	1500	1500
K_2HPO_4	40	40
$MgSO_4 \cdot 7H_2O$	75	75
$CaCl_2 \cdot 2H_2O$	36	36
Citric acid	6	6
Ferric ammonium citrate	6	6
EDTA (disodium salt)	1	1

Na_2CO_3	20	20
Trace metal composition		
H_3BO_3	2.86	2.86
$ZnSO_4 \cdot 7H_2O$	0.222	0.222
$Co(NO_3)_2 \cdot 6H_2O$	0.0494	0.0494
$MnCl_2 \cdot 4H_2O$	1.81	1.81
$CuSO_4 \cdot 5H_2O$	0.079	0.079
$NaMoO_4 \cdot 2H_2O$	0.39	0.39
Additional		
HEPPSO	7160	7160
Yeast Nitrogen Base (YNB), without amino acids, whithout ammonium sulfate	–	360[8], 1200[13]
pH 8.3 titration agent	KOH	KOH
KPO_3	–	118
Sucrose (for heterotrophs only)	–	13690

Table 1: Media composition of BG-11 and CoY^{BG-11}. Given concentrations of yeast-nitrogen base concentrations in CoY^{BG-11} are derived from published resources[8,12,13].

2. Tools and methodology to monitor the growth of co-cultures

NOTE: This protocol is a guideline for co-culture analysis and monitoring, from simple but work-intense techniques like microscopy and counting chambers to high-throughput applications like particle counters and single-cell flow cytometry. Apart from the actual co-culture, it is advisable to include axenic cultures of the single microorganisms to allow for a comprehensive analysis. As a general starting point for analytics, determine the OD of the cultures. In this section, different methodologies are detailed that can be used to convert relative measures of cell density (i.e., OD) into absolute values of cell number per volume. OD_{750} measurement is often used to determine the cell density of cyanobacteria cultures (due to absorption of 400-700 nm wavelengths), while OD_{600} is used for heterotrophic organisms. Both measurements provide approximate guide values with arbitrary units. Note that values can differ strongly between instruments.

1. Microscopic quantification with counting chambers

   NOTE: Counting chambers (i.e., Neubauer (improved)/ hemocytometers) are a simple and inexpensive means to determine the composition of co-cultures composed of cells that can be easily distinguished morphologically, *i.e.*, by their cell shapes (**Figure 1A**). It consists of a thick glass microscope slide and a cover glass. If the cover glass is positioned correctly on top of the slide, it creates two precision volume chambers with engraved grids (**Figure 1B-D**). By counting a defined area of a grid, the cell concentration of a suspension can be calculated. Counting chambers are available in different depths. Depending on the cell thickness, a suitable chamber depth should be chosen. For fungal cells such as *U. maydis* or *S. cerevisiae,* a depth of 0.1 mm works well. This chamber depth is too large for smaller cells such as cyanobacteria, and the cells are floating in the chamber. A chamber with a depth of 0.02 mm is suitable for both cyanobacteria and co-cultures of cyanobacteria and fungi.

   1. Ensure that the counting chamber and the cover glass are free from dust and cells.

      NOTE: Cleaning with 70% (v/v) ethanol and lint-free tissues is recommended directly before use.

   2. To position the cover glass correctly, slide it onto the two support bars with a little pressure, but be cautious to avoid breakage.

      NOTE: A specific, thick cover glass is required for this application (refer to chamber manual). Since the cover glass sometimes breaks during the assembly, it is good to have replacements at hand.

   3. When the cover glass is properly positioned, observe the so-called Newton's rings (**Figure 1B**) between the two glass surfaces, and that the cover glass does not slip anymore.

      NOTE: A gentle breath on the cover glass prior to the assembly often improves the result.

   4. Mix the cell suspension thoroughly and apply a few microliters (~2-10 µL depending on chamber depth) of the cell suspension to the edge of the chamber and allow it to fill completely via capillary force. Use an appropriate dilution of the cell suspension to allow reliable counting, e.g., resulting in 20 to 200 cells per large square (**Figure 1D**, red rectangle).

   5. Use an appropriate (e.g., 10x) objective of a light microscope (bright field or phase contrast mode) and focus on the counting chamber grid lines. Orientation is provided by the grid. Start counting the cells in the squares suitable for the given cell size.

      NOTE: Use a technical aid such as a hand-held counter or an appropriate smartphone application for counting (e.g., general "thing counter" or specialized hemocytometer apps providing extremely helpful features). Define a rule for counting cells located on the grid borders to avoid double counts. For example, cells located on the top and left borders of each square may be counted while excluding those on the right and bottom borders. Alternative rules exist[16]. If a fluorescence microscope is used, the cells can also be distinguished based on their autofluorescence or fluorescent markers.

   6. After counting, for example, all four large squares in the corners, determine the mean value for each cell type. With these mean values, the area of the utilized square and the depth of the given chamber (consult manufacturers' information), calculate the cell concentrations (see **Representative Results**).

NOTE: Depending on the cell size, different squares can be used for counting. For yeast and cyanobacterial cells, the four large squares in the corners are working well (**Figure 1D**, red rectangle).

7. After use, clean the chamber considering the requirements of the samples (e.g., inactivate genetically modified organisms appropriately).

2. Quantification using particle counters

NOTE: Depending on the characteristics of the cells in a co-culture, a particle counter can be applied to determine the cell numbers of the partners. For the applicability of this method, the cells need to differ strongly in their size; e.g., discrimination of bacterial and yeast cells can be achieved. Axenic cultures of the single microorganisms of the consortium need to be included to allow interpretation of results. Co-cultures harboring cells with similar sizes or more than 2 partners should be analyzed with the methodology described in sections 2.1 or 2.3.

1. Preparations

1. Adjust the OD of the culture to 0.1 and dilute the sample 1,000-fold with an isotonic measuring buffer (consult device manual) in a total volume of 10 mL. Prepare technical triplicates of all samples.

2. Select a suitable pore size of the capillary for the experiment.

NOTE: The pore size should be within the range of the smallest cells in the co-culture, but it should also include the larger cells. Do not go too small because the pore might clog due to cell aggregation, which can occur due to cell division. Available pore sizes differ. A capillary of 45 µm, for example, starts detecting cells with a diameter of 0.7 µm. (*U. maydis* and *S. cerevisiae* 3.5 µm-5.5 µm, *S. elongatus*/*Synechocystis* sp. ~1-2.5 µm).

2. Quantification of cells in a particle counter

1. Start the device. Eventually, perform a self-test for quality control (consult the manual of the specific device).

2. Load the samples into the sample cups, secure the lid, and mix the sample by slightly tilting the cup.

NOTE: Try to avoid foaming and bubbles. Always use one cup for each measurement. The cups can be cleaned and reused multiple times. Discard cups if there is any residue that cannot be removed. If the sample remains in the cup for longer periods, mix it again before measuring.

3. Record in a range of 0-30 µm due to cell aggregates potentially being larger than the single cells.

NOTE: First, use each of the axenic cultures of the co-culture partners to get an idea of the size of the cells and to determine if they can be distinguished by their size.

4. After evaluation, determine the composition of the co-culture in the same manner.

3. Consortium quantification using single-cell flow cytometry

NOTE: Single-cell flow cytometry is a high-throughput method that can be employed to determine cell numbers of individual partners in the co-culture, given that the partners can be discriminated by size/their light

scattering properties or/and fluorescence. Importantly, for method establishment axenic cultures of the co-culture partners are needed to analyze their properties in the cytometer and adjust the population gates accordingly. For strains carrying a fluorescent reporter, strains without the fluorescent reporter are needed as negative controls. A basic familiarity with cytometer applications will facilitate the successful implementation of the protocol below[17,18,19,20,21]. In this example, an artificial mixture of 3 microorganisms is analyzed (see **Representative results**): *Synechocystis* (expressing a cytosolic reporter fluorophore, mVenus) and the two yeasts *S. cerevisiae* and *U. maydis*. For a precise discrimination of *S. cerevisiae* and *U. maydis*, fluorescence markers have been introduced in the form of cytoplasmic green fluorescent protein (GFP) (*U. maydis* eGFP) and red fluorescent protein (RFP) mKate2 (*S. cerevisiae* mKate2) using basic molecular cloning techniques. If possible, strains with genomic integration of reporters should be preferred to avoid the requirement for continuous selection pressures.

1. Sample preparation
    1. Measure the OD of the cultures at a given growth stage to be analyzed.
    2. Adjust the OD of the samples to a range suitable for the measurement. Use an approximate OD_{750} of 0.05-0.5 for the cyanobacteria and an OD_{600} of 0.2-1.5 for the yeast using fresh culture medium (final volume: at least 500 µL). For co-cultures, aim for an OD_{750} between ~0.1 and 0.5 and record the dilution factor. For calculation, apply the formula provided in step 1.1.3.1. This will ensure that the cell count stays in a range of 1,000 - 10,000 cells/s at a flow rate of 10 µL/min (equivalent to 6,000 - 60,000 cells/µL).

        NOTE: For dilution, media or buffer without autofluorescence and particles can be used (e.g., BG-11). Be careful with media containing complex ingredients such as yeast extract that might show up as particles in the measurement.
    3. Transfer 300 µL of each sample cell suspension to an individual well in a 96-well-plate (standard round well, clear, flat bottom).

        NOTE: Alternatively, transfer 0.5-2 mL to a fluorescence-activated cell sorting (FACS) tube if the semi-automatic sample mode is used instead of the plate reader mode.
2. Cytometry measurement
    1. Start the cytometer and perform the startup program and quality control (QC) according to the manufacturer's instructions.
    2. Load (plate loader or semi-automatic sampling) and run the samples. Start with the control samples using the axenic cultures of each co-culture partner to identify each species based on their properties before aiming to separate them in a co-culture sample.
    3. Open dot plots and/or histograms for the fluorescence and scatter (e.g., forward scatter [FSC] or side scatter [SSC] height [-H] or area [-A]) channels relevant for the samples.
    4. Look for the required cell population(s) and adjust the threshold to exclude the technical noise and small particles from the medium.

NOTE: It is recommended to search for the cell population in an FSC-H over FSC-width or FSC-H over SSC-H dot plot and adjust the scale from linear to logarithmic for small cells like bacteria (typically 1-3 µm). Set the threshold in the FSC-H or SSC-H channel. For cells with less than 1 µm diameter, use the violet SSC (VSSC) in the violet laser at 405 nm for a better resolution.

5. Adjust the gain for each channel of interest manually to a range of 25-2,500. Adjust the gains such that the negative control (e.g., without the fluorescent marker) is in a range of 1×10^2-1×10^3 and the positive control (e.g., with the fluorescent marker) is in a range of 1×10^5-1×10^6 to get the best separation and stay within the range of 1×10^1-1×10^7.

   NOTE: The standard gains from QC can also be kept as a good reference.

6. To determine the cell concentration in the sample, record a defined sample volume using the record function (e.g., 10 µL at a flow rate of 10 µL/min). Later, divide the individual cell counts measured for this volume by the recorded volume to get a cell count per µL for each population.

   NOTE: Keep the initial dilution factor in mind when calculating the cell concentration in the culture/sample.

3. Gating/selection of cell populations

   NOTE: Independent of the device used to run the measurements, the analysis and gating can be performed in the cytometer operating software or commercial analysis software as exemplified below[22]. Additionally, analyzing flow cytometry data using nonproprietary software can be efficiently done with various open-source tools such as FlowCytometryTools[23] or FlowKit, utilizing FlowUtils[24] packages in Python. In the spirit of open science, this protocol shares an exemplary JupyterNotebook showcasing the use of these packages for basic data exploration. Of course, alternative software can be equally suited to perform flow cytometry bioinformatics[25].

1. Select certain cell populations by drawing gates around them in a dot plot or setting divider or line segments around peaks in a histogram (**Figure 4**).

   NOTE: The auto-gate function of the program can be used as a reference.

2. Separate the signals for photo- and heterotrophic organisms based on the autofluorescence of the phototrophic organism (mainly caused by chlorophyll) in the red region of the spectrum. Example: Use the histogram of the APC-H channel (excitation (ex.) at 638 nm, emission (em.) at 660/10 nm) and set a vertical divider between the right peak (phototrophic = chlorophyll autofluorescence positive population) and left peak/s (heterotrophic = chlorophyll autofluorescence negative population) (**Figure 4A**).

   NOTE: Fluorescent markers can also be used to distinguish two (or more) phototrophic partners within the same culture (e.g., mVenus for *Synechocystis*).

3. Separate the two heterotrophic organisms based on their fluorescent markers or scattering properties (FSC, SSC, if possible).

   NOTE: Separation based on scatter properties is only applicable if the cells have, for example, different cell size or morphology that results in visible changes in the scattering (e.g., *Synechocystis*: spherical cells with ~1.5-3 µm diameter vs. *U. maydis* elongated cells with a length of 10 µm and about 1-2 µm diameter[26]).

4. Example: Use the histogram of the FITC-H channel (ex.: 488 nm, em.: 525/40 nm) displaying only the "heterotrophic" population to distinguish between the heterotrophic cells containing GFP (e.g., *U. maydis* eGFP) and the ones without GFP (e.g., *S. cerevisiae* RFP/mKate2) (**Figure 4B**). Alternatively, use the histogram of the PC5.5-H channel (ex.: 561 nm, em.: 710/50 nm) displaying only the "heterotrophic" population to distinguish between the heterotrophic cells containing RFP (e.g., *S. cerevisiae* RFP/mKate2) and the ones without RFP (e.g., *U. maydis* GFP)(**Figure 4C**).

   NOTE: Keep in mind that two (or more) different cell types can "stick" together during measurement. These "doublets" or "multiplets" can easily be detected in dot plots of both fluorescent markers (e.g., APC-H over FITC-H to identify doublets of phototroph and GFP-tagged heterotrophic partner).

5. For basic data analysis in Python, follow the instructions in this Jupyter (https://git.rwth-aachen.de/computational-life-science/cytoflow).

   NOTE: This notebook supports three steps of data analysis. i) Import data: Import of FCS files. ii) Perform quality control: Check for data integrity, and number of events. iii) Visualize data: Use plots like histograms, scatter plots, and density plots to visualize data. In addition, the procedure how to perform gating to isolate populations of interest reproducing **Figure 4** is displayed.

   To facilitate analysis of co-cultures in long-term projects, the researcher is recommended to use the above methods to count cell numbers and create standard curves to compare OD values to absolute cell counts. Optical density is a relative measurement that can vary based on species, strain, growth conditions, and spectrometer. Therefore, the generation of "in-house" standard curves is necessary for the accurate conversion of OD values to cell numbers.

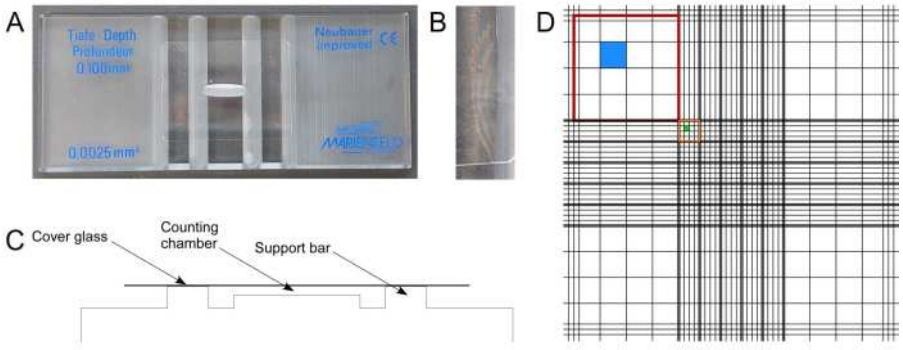


Figure 1: Microscopic quantification of morphologically distinguishable cells. (**A**) A Neubauer counting chamber. (**B**) Newton's rings indicate the correct positioning of the special cover slip. (**C**) Schematic depiction of the chamber architecture with the central cavity of defined volume for cell counting. (**D**) The grid of the depicted Neubauer counting chamber consists of nine large squares with a size of 1 mm^2 (red). The four large squares in the corners are further divided into 16 squares (blue). The central large square is divided into 25 group squares with a size of 0.04 mm^2 (orange). Each group square consists of 16 smallest squares (green). The figure was generated based on publicly available manufacturers' information.

Please click here to view a larger version of this figure.

Representative Results

Establishment of co-cultures of phototrophic *S. elongatus* and heterotrophic yeasts

We have previously reported detailed results from the co-cultivation of *Synechococcus elongatus* PCC 7942 with a variety of substrains of *S. cerevisiae*. For a comprehensive description of co-culture results with this cyanobacteria/yeast pair, see[8]. For the sake of brevity and accuracy, these results are not reproduced here. Briefly, prior results indicate a number of considerations that are important for the establishment of long-term cyanobacterial-yeast co-cultures. Of primary concern, the capacity of yeast to survive under conditions where cyanobacteria provide the sole forms of fixed carbon is strongly dependent upon the efficiency with which the yeast strain can utilize sucrose. *S. cerevisiae* strains that were evolved or engineered to more efficiently metabolize sucrose[27,28] were more likely to survive the transition to the co-culture growth mode, achieved higher cell densities, and exhibited higher robustness in long-term (days to weeks) cyanobacterial co-culture experiments[8]. The initial phase of inoculating a co-culture was especially important for yeast viability, likely due to stresses of culture dilution, switching media composition, and/or withdrawal of a more concentrated carbon source. Therefore, efforts to ease the transition from a richer growth medium to minimal carbon availability at co-culture initiation can improve experimental performance and consistency (see step 1.2.1.1). Additionally, *S. cerevisiae* exhibited stress responses consistent with hyperoxia when inoculated into dense cultures of *S. elongatus*, consistent with the formation of O_2 as a primary byproduct of

oxygenic photosynthesis. Therefore, efforts to prevent the overgrowth of the cyanobacterial partner and/or alternating "day/night" light cycles could substantially extend the viability of *S. cerevisiae* in long-term co-cultures. See the Discussion section for an additional summary of phenomena common in co-cultures relative to axenic control samples.

Monitoring the growth of co-cultures using different methodologies

In the following section, the exemplary quantification of an artificially mixed tripartite consortium of *Synechocystis* and the two yeasts *S. cerevisiae* and *U. maydis* using three different methods is described. For the mixture, OD_{750} (for cyanobacteria) and OD_{600} (for the heterotrophs) of single cultures were determined and adjusted to OD 0.1. Single cultures were mixed in a ratio of 1:1:1 using optical density (which is distinct from cell counts, see above). To facilitate the discrimination of the yeasts in the cytometer, reporter strains of genetically modified *S. cerevisiae* FY1679-O1B[29] constitutively producing cytoplasmic mKate2 (genotype: URA3Δ/pTDH3::mKate2; strain: *S. cerevisiae* mKate2[30]) and *U. maydis* strain AB33[31] constitutively producing eGFP (genotype: pep4Δ/pRpl40::egfp; strain: *U. maydis* eGFP[32]) were used. Of note, the cyanobacterial strain was equipped with a replicative plasmid promoting the constitutive, strong expression of the yellow fluorescent protein version mVenus (*Synechocystis sp*. PCC 6803 pSHDY-Pcpc560-mVenus, strain *Synechocystis* mVenus, similar to[33]) and in addition, exhibits the typical strong autofluorescence due to the presence of the photosynthetic machinery.

Microscopic quantification using counting chambers: All cell types in the used artificial mixture of three microorganisms can easily be discriminated microscopically (**Figure 2A**): *Synechocystis* and *S. cerevisiae* are represented by spherical cells that however differ greatly in their diameter (*Synechocystis*: approx. diameter of 1.5-3 µm, *S. cerevisiae*: approx. diameter of 3-6 µm), whilst *U. maydis* cells have an elongated, cigar-shape morphology and a length of at least 10 µm (**Figure 2A**). These clear morphological traits allow for the exact quantification of each partner in the mixture. As an illustrative example, 37, 18, 36, and 21 *S. cerevisiae* cells are counted in the four large squares, respectively (**Figure 2B**). The mean value is 28 cells. Since a large square of the used hemocytometer has an area of 1 mm^2 and the chamber depth was 0.02 mm, this results in 28 cells per 0.02 µL. This corresponds to 1,400 cells/µL, which is equivalent to 1.4 x 10^6 cells/mL. The other cell types were counted, and concentrations were determined accordingly (**Figure 2C**, **Table 2**).

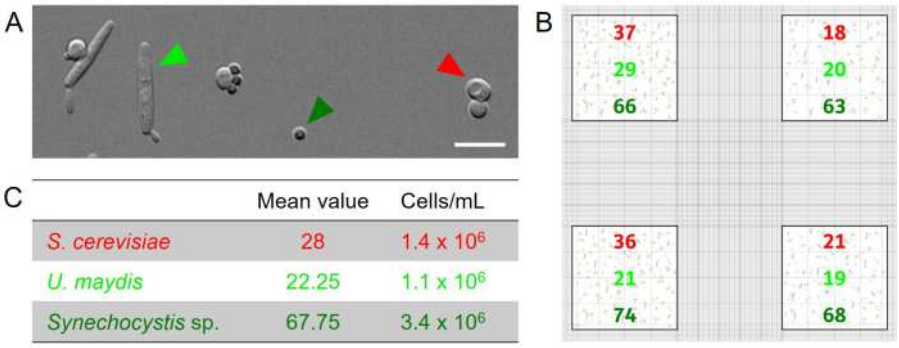


Figure 2: Microscopic quantification of a tripartite consortium consisting of *Synechocystis* and the yeasts *S. cerevisiae* and *U. maydis*. (**A**) Microscopic differential contrast (DIC) image of an artificially combined mixture of the indicated microorganisms. All species can be easily distinguished by their morphology. Scale bar: 10 µm. (**B**) Exemplary counting results for an artificially assembled mix of the indicated strains based on axenic cultures with an OD of 0.1. The four large squares at the grid edges were analyzed (red mark in **Figure 1D**). (**C**) The mean value of the different cells counted in the four squares were used to calculate the concentration of cells in the suspension using the following equation: Mean value/(chamber depth [0.02 mm] x size of counted square [1 mm^2] x 1,000). Please click here to view a larger version of this figure.

Quantification using particle counters: Particle counters determine the number of particles in a suspension depending on their size. In the example dataset, a particle counter with a 45 µm capillary was used. Since *S. cerevisiae* and *U. maydis* cells show similar dimensions, they cannot be distinguished in the particle counter, while the smaller *Synechocystis* cells can be clearly separated. The analysis of single cultures hence shows peaks at identical positions referring to 3 to 6 µm for the two yeasts (**Figure 3A**). During the measurement, a negative pressure is applied, which causes the cells to enter the capillary. This briefly changes the electrical resistance so that the device can determine the particle size based on the alteration. In the mixed culture, two distinct peaks can be detected, one associated with the smaller cyanobacterial cells and the other representing a joint fraction of the two yeast species (**Figure 3B**). Importantly, in addition to the peaks reflecting the living cells, additional peaks were detected at about 1 µm (**Figure 3**), corresponding to cell debris and smaller particles. Signals that would appear at larger diameters than expected can be caused by cell aggregations. Of note, the shape of peaks reflects the homogeneity of cells: A sharp peak shows very homogenous cells, which is unlikely for a co-culture. In a co-culture, it is expected to see a very broad peak or even two peaks if the partners of the co-culture are clearly different in size. For the axenic cultures of heterotrophic fungi, the peak might be a medium broad peak.

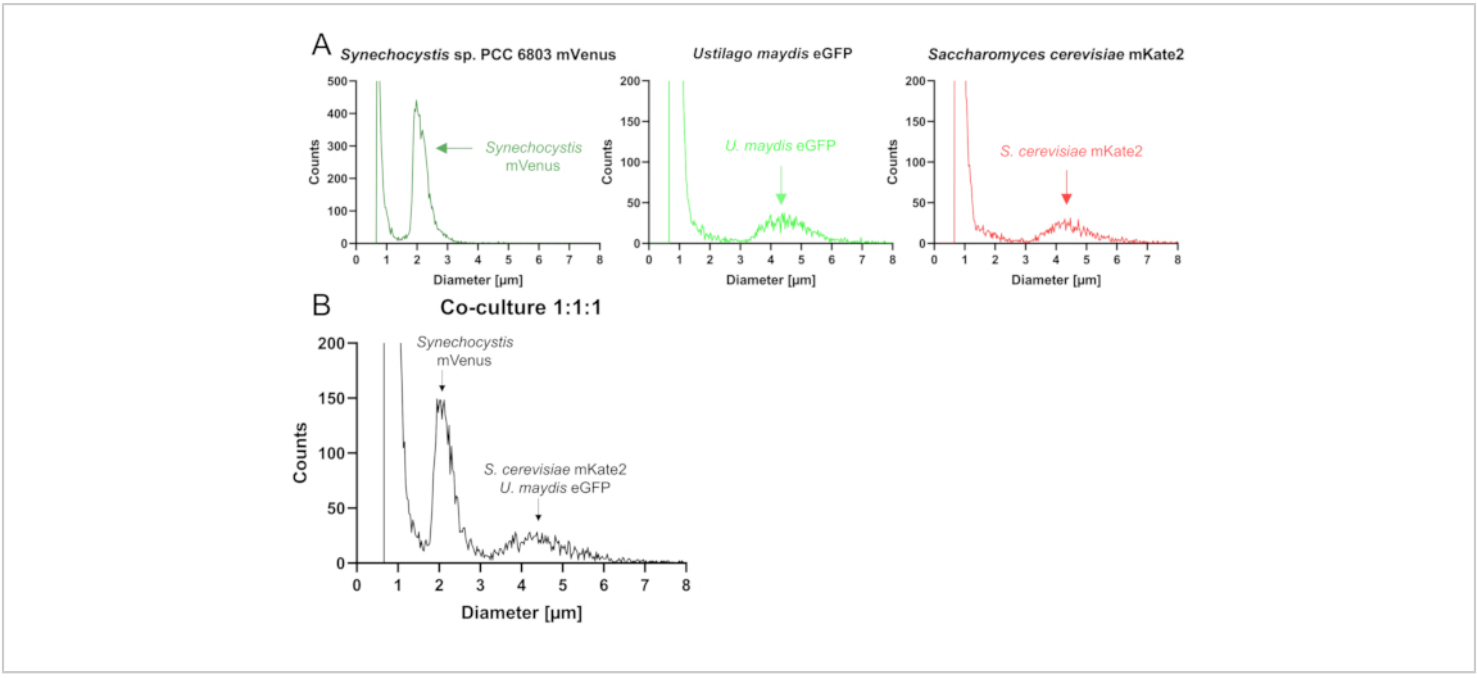
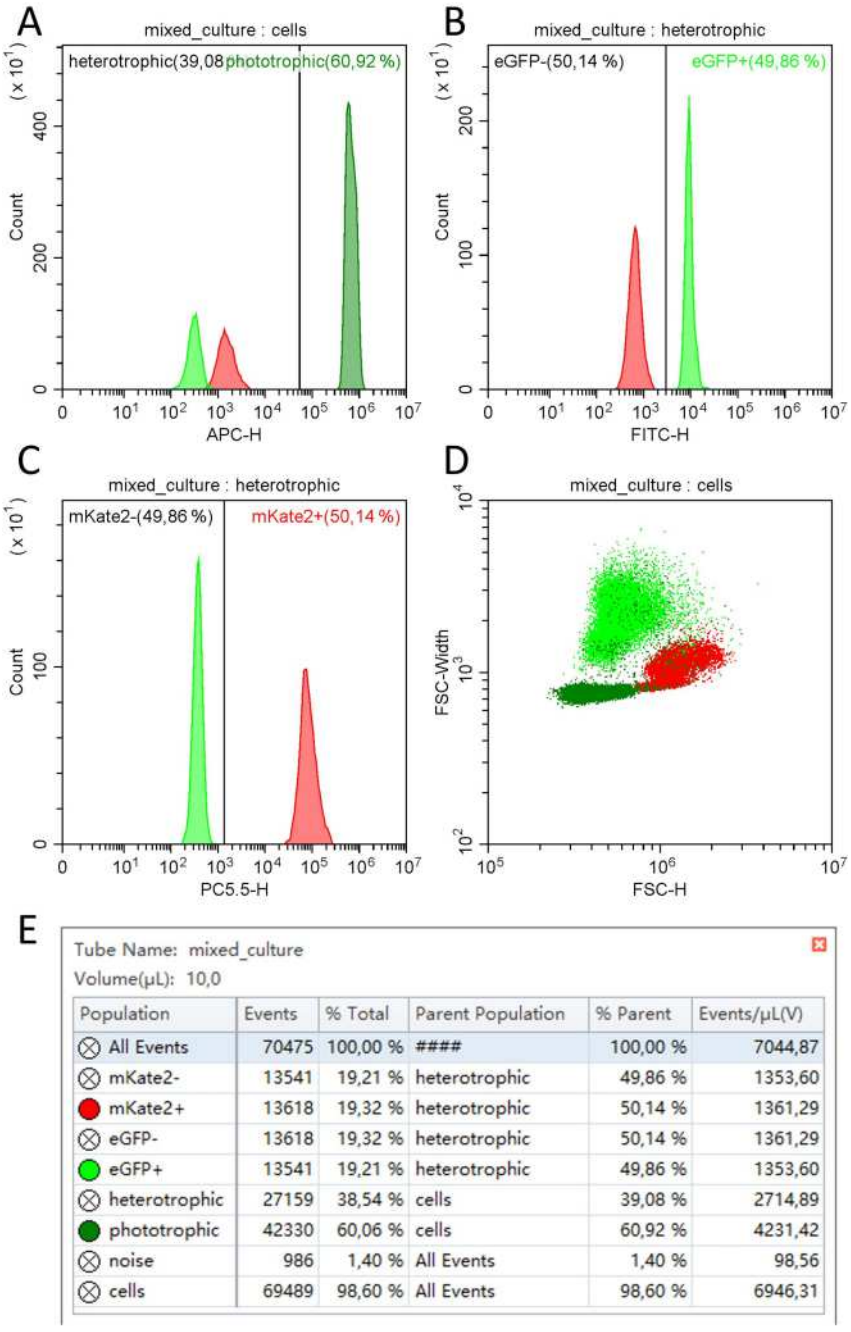


Figure 3: Quantification of a tripartite consortium consisting of *Synechocystis* mVenus and the yeasts *S. cerevisiae* mKate2 and *U. maydis* eGFP using a particle counter. (**A**) Visual output of analyses of axenic cultures of the three strains as indicated in the graphs (cell counter with 45 µm capillary). (**B**) Exemplary visual output file for the analysis of the artificial tripartite consortium using identical conditions. The particle counter does not support the discrimination of the two yeast species which are both represented by the second peak. Please click here to view a larger version of this figure.

Quantification using single-cell cytometry: Using the single-cell flow cytometer the different cell populations can easily be distinguished based on their (auto-) fluorescence and light scattering properties. Phototrophic cells (*Synechocystis*) can be differentiated from heterotrophic cells (*U. maydis* and *S. cerevisiae*) based on the red autofluorescence of the photosynthetic pigments which is measured in the APC-H channel (**Figure 4A**). Based on that initial separation of phototrophic and heterotrophic cells, the two heterotrophic populations can be distinguished based on their fluorescent markers eGFP in the FITC-H channel and mKate2 in the PC5.5 channel (**Figure 4B**,**C**). In a dot plot showing the scattering properties of all three populations (FSC-H and FSC-Width), the populations can also be distinguished with only some minor overlaps of the populations (**Figure 4D**).

With this method, 10 µL of the diluted samples were analyzed with a flow rate of 10 µL/min, allowing a quantification of approximately 70,000 cells in 1 min. Roughly 60% of those cells could be assigned to *Synechocystis* (4.23 x 10^6 cells/mL), while the remaining 40% were equally distributed between *U. maydis* (1.35 x 10^6 cells/mL) and *S. cerevisiae* (1.36 x 10^6 cells/mL, **Figure 4E**, **Table 2**).



E

Tube Name: mixed_culture
Volume(µL): 10,0

Population	Events	% Total	Parent Population	% Parent	Events/µL(V)
All Events	70475	100,00 %	####	100,00 %	7044,87
mKate2-	13541	19,21 %	heterotrophic	49,86 %	1353,60
mKate2+	13618	19,32 %	heterotrophic	50,14 %	1361,29
eGFP-	13618	19,32 %	heterotrophic	50,14 %	1361,29
eGFP+	13541	19,21 %	heterotrophic	49,86 %	1353,60
heterotrophic	27159	38,54 %	cells	39,08 %	2714,89
phototrophic	42330	60,06 %	cells	60,92 %	4231,42
noise	986	1,40 %	All Events	1,40 %	98,56
cells	69489	98,60 %	All Events	98,60 %	6946,31

Figure 4: Quantification of a tripartite consortium consisting of the cyanobacterium *Synechocystis* mVenus and the yeasts *U. maydis* eGFP and *S. cerevisiae* mKate2 using single-cell flow cytometry. Example plots obtained after measurement of a mixed culture of *Synechocystis* mVenus (dark green), *U. maydis* eGFP (light green) and *S. cerevisiae* mKate2 (red) in a ratio of ⅓ OD_{750} / ⅓ OD_{600} / ⅓ OD_{600} on a cytometer (**A**) showing a histogram of the event count and fluorescence in the APC-H channel (ex.: 638 nm, em.: 660/10 nm) of all cells used to differentiate phototrophic and heterotrophic cells based on their autofluorescence. (**B**) A histogram of the event count and fluorescence in the FITC-H

channel (ex.: 488 nm, em.: 525/40 nm) of all heterotrophic cells used to differentiate *U. maydis* eGFP and *S. cerevisiae* mKate2 cells based on their green fluorescence properties. (**C**) A histogram of the event count and fluorescence in the PC5.5-H channel (ex.: 561 nm, em.: 710/50 nm) of all heterotrophic cells used to differentiate *U. maydis* eGFP and *S. cerevisiae* mKate2 cells based on their red fluorescence properties. (**D**) A dot plot of the scattering signals in the FSC-Width over the FSC-H channel used to identify the cell populations without fluorescence properties. (**E**) The population statistics including the event counts, percentages, and arithmetic means and standard deviations (SD) of the relevant fluorescence channels. The total amount of cells, along with their size and fluorescence, was determined in a volume of 10 µL with a flow rate of 10 µL/min. Please click here to view a larger version of this figure.

To comparatively visualize the output of the different quantification methods the determined cell numbers are presented in the following two tables (**Table 2** and **Table 3**). The final concentrations of cells determined using the three previously described methods are in a similar range for all methods. The cytometer provides the highest sample size, followed by the particle counter and the microscopic quantification, with a decrease of approximately one order of magnitude between the methods.

Co-culture 1:1:1	**Photometer**	**Cytometer**		**Particle Counter**		**Microscopy Counting Chamber**	
Organism	$OD_{750/600}$	cell count	cells/mL	cell count	cells/mL	cell count	cells/mL
***Saccharomyces cerevisiae* mKate2**	0.0333	13,618	1.36×10^6	1,546*	2.58×10^6*	112	1.40×10^6
***Ustilago maydis* eGFP**	0.0333	13,541	1.35×10^6			89	1.11×10^6
***Synechocystis* sp. PCC 6803 mVenus**	0.0333	42,330	4.23×10^6	3,094	5.16×10^6	271	3.39×10^6

Table 2: Comparison of the different quantification methods: Artificial mixed culture. Note that *U. maydis* and *S. cerevisiae* cannot be distinguished using a particle counter (*).

Single culture	Photometer	Cytometer		Particle Counter		Microscopy Counting Chamber	
Organism	$OD_{750/600}$	cell count	cells/mL	cell count	cells/mL	cell count	cells/mL
***Saccharomyces cerevisiae* mKate2**	0.1	38,936	3.89×10^6	1,928	3.21×10^6	403	4.03×10^6
***Ustilago maydis* eGFP**	0.1	36,927	3.69×10^6	2,465	4.11×10^6	307	3.07×10^6
***Synechocystis* sp. PCC 6803 mVenus**	0.1	127,864	1.28×10^7	8,186	1.36×10^7	428	1.07×10^7

Table 3: Comparison of the different quantification methods: Calibration with single/axenic cultures.

Discussion

Handling of microorganisms in single axenic cultures in a laboratory context has been established for decades for many microbial models. Yet, though the prevailing form of life in nature is microbial communities, the combination of two or more partners in a single cultivation vessel is less established, and challenges are presented by gaps in the existing knowledge and methodology. It is also more difficult to predict the behavior of cells in a community, as emergent interactions and metabolite exchange arise between the cells, strongly influencing the fate of the co-culture[34,35]. Hence, co-culture establishment is not trivial, including on the level of growth media definition, the identification of common growth conditions, interspecies exchange of trace metabolites/ signals, and the resulting co-culture composition over time. Progress of the last years in the assembly of phototrophic, sugar-secreting cyanobacteria with heterotrophic partners now allows to deduce first rules and methodology that can provide a helpful guideline to the design of novel co-cultivation pairs. Based on that knowledge, in the first part of this protocol a step-by-step guideline to the assembly of co-cultures containing a sucrose-secretion cyanobacterium and one or more yeasts is provided.

One critical consideration when first attempting to establish a co-culture between unrelated microbes is the composition of a common growth medium that satisfies all nutrient requirements for the two or more species. Due to space limitations, it is not feasible to provide a fully detailed protocol for this process here, which may also require a high degree of customization in some instances, but instead the following outlines important considerations to bear in mind. One straightforward initial approach involves comparing the typical cyanobacterial growth medium (e.g., BG11; see **Table 1**) with any established minimal media composed for the heterotrophic species of interest. Supplementing the standard cyanobacterial medium with any missing components that are contained within the heterotrophic minimal medium is a good starting point for initial testing[8,36]. Bear in mind that because minimal media are often supplemented with a significant organic carbon source for heterotrophic growth (e.g., 1%-4% glucose), they are often designed to support higher heterotroph cell density than is likely to be achieved in initial co-culture experiments. Likewise, some

common medium components can also act as an organic carbon source independently of the photosynthate provided by the cyanobacterial partner (e.g., citrate), which can complicate later analysis of cyanobacteria/heterotroph co-cultures. Therefore, it may not be necessary to complement the minimal cyanobacterial medium with the full concentration of missing elements when designing a co-culture medium. For example, many organisms vary in their efficiency of use of different forms of environmental nitrogen (e.g., N_2, nitrate, nitrite, urea) and may be completely unable to utilize some of the more oxidized nitrogen sources. Similarly, many microbes may require supplementation of essential vitamins (e.g., vitamin B_{12}), co-factors or essential amino acids because they lack complete biosynthetic pathways for direct synthesis of these compounds. For these reasons, it may be most practical to start by simply "merging" the established minimal medium of the cyanobacterial partner (e.g., BG11) together with a well-defined minimal medium of the heterotroph (e.g., synthetic defined [SD]). Later cycles of reiteratively removing/reducing superfluous components can be used to optimize the medium and reduce the abundance of any compounds that may be inhibitory to the growth of one of the partners. A useful starting point is to buffer the medium at a neutral or slightly basic pH, as these tend to be conditions favored by most cyanobacterial model species.

At this point, it is often helpful to conduct preliminary tests of the growth of the supported heterotroph in the new co-culture medium when an excess of sucrose is supplied. Of course, when selecting a potential heterotroph, it is important to pick one that is capable of catabolizing the primary source(s) of organic carbon that will be supplied by the cyanobacterial partner. It is useful to note here that sucrose, as the dominant carbohydrate supplied in many engineered cyanobacterial/heterotrophic cultures[4], is not a carbohydrate that is as universally utilized by heterotrophic microbes as glucose: dedicated sucrose transporters must be encoded by the heterotrophic species or extracellular invertases may be necessary to convert sucrose to fructose and glucose that are often recognized by higher-affinity transporters[8]. An important observation commonly reported by multiple laboratories researching mixed microbial communities is that higher-order synergies and antagonisms emerge between the phototrophic and heterotrophic partners[4,8,13]. For example, other naturally secreted metabolites (e.g., organic acids, reduced forms of nitrogen) or co-factors (e.g., siderophores) may enable higher growth rates of one or both partners when cultivated in the same medium relative to axenic controls. Conversely, potentially harmful metabolic byproducts, such as hyperoxygenation of the medium by photosynthetic water splitting, have been reported to cause inter-species inhibition of growth for one or both partners[8]. Therefore, axenic controls can provide a useful benchmark, but the co-culture performance may vary from expectations due to these emergent properties.

Depending on the heterotrophic partner and the capabilities of *S. elongatus* to excrete sucrose (based on the level of induction, used IPTG concentration), different ratios of both organisms should be tested. The success of the cultures depends primarily on the ability of *S. elongatus* to maintain the growth of the heterotrophic partner (i.e., can it produce enough carbon source). While overgrowth of the heterotrophic strain is typically limited by the lack of organic carbon provided in the co-culture medium composition, cyanobacteria can outpace the heterotroph, which may lead to emergent inhibitory interactions (e.g., hyperoxic conditions[8,13]). When attempting to initially determine appropriate ratios of cell density for the cyanobacterium: yeast, a good approximate rule is that the cyanobacterial

partner can support an equal cell volume of the accompanying heterotrophic cells. Since eukaryotic yeasts tend to have considerably larger cell volumes than model sucrose-secreting cyanobacteria, this may likely mean that the density of cyanobacterial cells will be considerably higher (e.g., 50-100 fold for *S. cerevisiae*) in a steady-state. Therefore, a good starting point when setting up a new co-culture with the researcher-specific laboratory and species conditions would be to calculate the average cell volume for both the cyanobacterium and yeast (based on published values; e.g., see B10NUMB3R5[37]) to estimate the volumetric ratio. Initial flasks can be seeded with this ratio of cells. To explore the solution space, the researcher may wish to hold the concentration of cyanobacteria constant (e.g., OD_{750} = 0.3) while varying the concentration of yeast cells up or down by approximately an order of magnitude in increments based on the throughput allowed by the researcher's available phototrophic cultivation space. Of course, this volumetric 'rule of thumb' is dependent upon the rate at which the phototrophic partner is capable of secreting organic photosynthates (e.g., sucrose) that can be utilized by the heterotrophic partner. Once co-cultivation conditions have been established, careful monitoring of the growth performance of both partners over time will provide valuable data regarding ideal species ratios, especially if early co-cultures can be maintained for days to weeks, thereby allowing the researcher to identify the steady-state ratio reached near the end of a co-culture. This information can be utilized when selecting the initial inoculation density for each partner species in subsequent experiments to help the culture more rapidly reach the self-determined ideal species ratio.

In the second part of the protocol, detailed instructions for co-culture analytics are provided. A reliable quantification of the co-cultures is key to their successful implementation: Growth (or at least metabolic activity) of the phototropic, carbon-secreting partner is essential to sustain the heterotrophs. While the ratio of cyanobacteria:heterotroph used to inoculate the culture may not always be critical to optimize, since longer-term co-cultures tend to converge towards stable proportions, it may be important to integrate methods to check unrestrained growth of either partner through culture dilution or encapsulation of one or more species[36,38,39,40]. As mentioned above, byproducts of the cyanobacterial partner may be inhibitory to the heterotroph at high concentrations (e.g., O_2), and some products of heterotrophic metabolism may also be detrimental to cyanobacterial health. Most published co-cultures utilize heterotrophs that have a faster growth rate than most model cyanobacterial species; therefore, the heterotrophic growth rate tends to be constrained by the supply of organic carbon produced by the cyanobacterium. Nonetheless, determination of the species abundance dynamics over time is a critical value for elucidating failures in stability and optimizing for robust co-cultures in long-term cultivation.

Protocols for quantification using counting chambers, particle counters, and single-cell flow cytometry are provided. All techniques are valuable tools for the characterization of co-cultures, however, with different prerequisites, advantages, and limitations. Counting chambers are very broadly applicable for co-culture quantification, provided that all partners in the culture can be distinguished visually by their cell shapes or other properties. The great advantage is the low price of this device, such that it is basically achievable for every laboratory. Besides information on the co-culture composition and ratio of the different partners, an impression of the cell's morphology and fitness can be gained alongside, and potential contaminants can be detected. However, the application of the counting chamber is also very time-

consuming, comes with a high workload, and can only support a low throughput.

Both particle counters and single-cell flow cytometry provide the huge benefits of a high throughput and convenient, time-saving handling. While the cell counter relies on clear differences in cell sizes of the partners in the co-culture, single-cell cytometry can also discriminate fluorescence labels, resulting in the ability to quantify co-cultures with more than two members of the same size or even two different mutants of the same organism based on different fluorescent markers. In the provided example of an artificial assembly of *Synechocystis* with *S. cerevisiae* and *U. maydis*, the intracellular fluorescence reporters mKate2 and eGFP were used to discriminate the two yeasts. The same fluorescent reporters could also be used to distinguish two genetically modified strains of the same organism (e.g. *S. cerevisiae*) which could not be separated based on their light scattering properties alone. Similar strategies have been implemented to track synthetic bacterial consortia[41]. Depending on the cell type, the number of partners in a consortium, and their potential range of autofluorescence(s), the selection of fluorescent markers needs extra attention to avoid overlap of spectral qualities, particularly given the autofluorescent properties of cyanobacteria.[42]

We advocate here how to adhere to FAIR guiding principles in terms of storage, management, and sharing of scientific data. FAIR is an acronym that stands for Findable, Accessible, Interoperable, and Reusable[43]. As these principles gain wider acceptance, they promise to transform the landscape of biological research, promoting a more open, collaborative, and efficient approach to data management and use. While critical components of accessibility can be ensured by depositing data on Annotated Research Context (ARC)[44], it is important to enable the reproduction of the data processing steps with open-access software. Raw FCS files store both data and metadata (information about lasers and detectors, including wavelengths, filters, etc.) about flow cytometry experiments[45]. They can be loaded and read outside of proprietary software that allows for sophisticated data analysis, e.g., a number of packages are available to work with raw data inside the Python environment. The developed package demonstrates how to load, read, and visualize raw .fcs files containing cytometry data outside the proprietary software using one of many available packages in Python[23]. Using open software such as described above eliminates the need for costly licensing fees associated with proprietary software, provides full control over data processing (e.g., transformation, compensation, gating) and additionally allows integration of numerous tools in one place.

A valuable extra benefit of utilizing single-cell cytometry in conjunction with a cell sorter is the opportunity to also collect cells of a distinct type by FACS. This can be extremely valuable for applying any -omics technologies for detailed insights into the species interaction within the co-culture, like RNASeq or metabolomics. The clear downside of these devices is their high prices, so the affordability and availability of such machines might be an apparent limitation in some laboratories.

Interestingly, a direct comparison of the presented methods for quantification of the three partners in a consortium revealed a very good accordance between the different techniques, indicating that the mixture that has been assembled based on OD measurements is reliably quantified by all described methods. Interestingly, the total number of cyanobacteria was about 3-4 times higher than the number of the two yeast species, a phenomenon likely due to their

smaller cell size. This observation is in good accordance with the common knowledge that ODs do not provide information on actual cell numbers without calibration[46]. The yeasts, however showed counts on a comparable level (**Table 2** and **Table 3**). In addition, the comparison with the quantification of the single cultures demonstrates that all three methods are reliable tools to discriminate the cell types, with the exception of the particle counter that did not separate the two yeast species.

In essence, single-cell cytometry applications are clearly the most powerful tool to monitor the composition of co-cultures, allowing for the counting of 1,000-10,000 cells per second. This is especially true if the number of partners increases to more than two or if the partners are similar in shape and/or diameter. Notably, there are plenty of alternatives that allow the monitoring of co-cultures. Growth of fluorescently labeled microbes in co-cultures can, for example, be tracked continuously by fluorimetry or microbioreactors[47]. However, these are often limited to a co-culture of two partners and require careful experimental design, for instance, in the choice of fluorescence markers. Amplicon sequencing (16S rRNA sequencing) and other next-generation sequencing techniques in combination with sophisticated bioinformatics is another option for the characterization of synthetic communities[48,49,50]. These techniques are suitable for high throughput approaches and can address established interactions in long-term cultivations, evolutionary questions, or tracking of mutations with multiple microbial partners.

Taken together, simplistic microbial co-cultures that are rationally designed provide a powerful "bottom-up" approach for interrogating inter-species dynamics that can be more difficult to approach within multi-species communities that dominate the natural world[51,52,53]. Herein, a streamlined protocol that may be readily adapted by the scientific community for the establishment and analysis of novel pairs of cyanobacteria and heterotrophic partners is provided. It is evident that much research is needed to both capitalize upon the potential fundamental insights that may be gained from artificial microbial co-cultures as well as to determine if synthetically designed microbial consortia can match the potential often ascribed to them in the literature for biotechnological applications.

Disclosures

The authors have nothing to disclose.

Acknowledgments

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - SFB1535 - Project ID 458090666 (project B03 to KS, AM and IA), and Major Research Instrumentation INST 208/808-1. DCD is currently visiting HHU Düsseldorf as a Mercator Fellow of the SFB1535. Additional support for EJK was provided by National Science Foundation Awards #1845463 and #2334680.

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