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RESEARCH

Development of a high-throughput label-free method for enrichment of antimetabolite-resistant yeast strains using pico-litre agarose beads

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Abstract Antimetabolites, which are structural analogues of metabolites or carbon sources that exhibit toxicity by disrupting metabolism, can couple phenotypes of interest to growth and thereby facilitate the selection of relevant strains from large mutant pools generated by random mutagenesis. Toxicity and high costs of many antimetabolites provide incentives to minimise liquid volume during cultivation, while still capturing the genetic diversity in large mutant pools. This study aims to explore the use of high-throughput microbead-based cultivation and label-free enrichment of antimetabolite-resistant mutants. The generation of a large number (~ 10^7 microbeads) of picolitre-sized cultivation compartments using water-in-oil emulsions enables high-throughput, parallel growth of millions of individual mutants in a total medium volume of just 0.3 mL. A reduction in screening medium volume reduces the quantities of expensive antimetabolites required. Employing a fluorescence-activated microbead-sorting-based approach enables label-free screening and selection of mutants based on autofluorescence signals as indicators for growth. The potential of this microbead-based, label-free selection method was demonstrated by enriching a G418 (geneticin) antibiotic-resistant and a glucose analogue (2-deoxyglucose) resistant Saccharomyces cerevisiae strains by 16,000-fold and 600-fold, respectively, in single-step enrichment experiments.

Key points
- *Small-volume cultivation cuts screening costs for costly antimetabolite resistance.*
- *Autofluorescence-based screening enables label-free detection of resistant mutants.*
- *10^4- and 10^3-fold enrichment of antibiotic- and glucose analogue-resistant strains.*

Keywords Microdroplets · High-throughput · Antimetabolite · Label-free detection · Yeast

Introduction

The throughput of classical strain improvement has significantly increased due to the implementation of high-throughput screening techniques (Zhou and Alper 2019; van Tatenhove-Pel et al. 2020; Zeng et al. 2020). Classical strain improvement involves random mutagenesis followed by mutant selection. This approach is particularly relevant when targeted metabolic engineering strategies are difficult to implement due to limited knowledge of responsible genes, lack of genetic engineering tools, and selection for complex, multigenic phenotypes such as stress tolerance. It is also crucial for the food fermentation industry, where regulatory and/or public acceptance issues limit the application of targeted genetic engineering (Marshall 2026; Bachmann et al. 2015; Marsit and Dequin 2015).

Classical strain improvement typically involves two key steps: (i) generation of genetic diversity, typically by random mutagenesis, and (ii) isolation of improved variants by screening or selection (Zhou and Alper 2019; Zeng et al. 2020). Use of random mutagenesis imposes a requirement for high-throughput screening methods that can identify, select, and enrich mutants with desired phenotypes from massive mutant pools (Zeng et al. 2020). When relevant phenotypes can be coupled to growth, higher specific growth rates and/or survival rates enable mutants of interest to dominate cultures upon propagation. For certain phenotypes, such as improved growth-coupled catabolic product formation rates and tolerance to environmental stress factors,

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adaptive laboratory evolution offers a straightforward way for growth-based selection (Bachmann et al. 2015; Mans et al. 2018; Zeng et al. 2020). It is much more challenging to adopt growth-coupled selection for mutants that generate higher titres, productivities or yields of non-growth-coupled assimilatory products (Mans et al. 2018). Challenges in implementing growth-coupled strategies also complicate the selection of strains with deregulated catabolite repression, which are highly relevant in industrial biotechnology.

Antimetabolites are compounds whose strong structural similarity to natural metabolites causes interference with metabolic or regulatory mechanisms in wild-type cells (Adrio and Demain 2006; Fiedurek et al. 2017; Cardoso et al. 2018). Resistance to antimetabolites have been widely applied to select for improved synthesis of non-growth-coupled products, and for alleviation of catabolite repression (Adrio and Demain 2006; Fiedurek et al. 2017; Cardoso et al. 2018). In many anabolic product pathways, one or more of the enzymes in the pathway are subject to feedback inhibition by the product or a downstream intermediate (Adrio and Demain 2006; Fiedurek et al. 2017). To overcome such feedback inhibition and thereby enable product accumulation, one effective approach is to render the target enzyme(s) insensitive to feedback inhibition by selecting for tolerance to antimetabolites that mimic the natural inhibitor (Adrio and Demain 2006; Fiedurek et al. 2017). Mutants tolerant to inhibition by the antimetabolite are typically also insensitive to feedback inhibition by the native inhibitor and therefore able to accumulate the product, whereas, non-tolerant mutants do not survive. Thus, resistance to antimetabolites provides an effective growth/no-growth selection strategy.

Antimetabolites can also be used to alleviate catabolite repression. For example, repression by glucose is a key factor in preventing simultaneous conversion of multiple sugars in feedstocks such as lignocellulosic hydrolysates (Jung et al. 2015; Gao et al. 2019; Fox and Prather 2020; Shrestha et al. 2023). Catabolite (glucose) repression-negative mutants of *S. cerevisiae* can be obtained by selecting for tolerance to the antimetabolite 2-deoxyglucose, a glucose analogue without a hydroxyl group at the second carbon, in the presence of another carbon source (Neigeborn and Carlson 1987; McCartney et al. 2014a; Soncini et al. 2020). Similar strategies are applied to deregulate nitrogen-source and phosphate-source repression for improved production of secondary metabolites, such as antibiotics (Adrio and Demain 2006; Fiedurek et al. 2017).

When selecting for antimetabolite-resistant mutants, spatial separation of mutants ensures that genetic diversity of mutant pools is maintained. If all mutants are grown in a single compartment, the fastest growing mutant would outcompete slower growing ones, allowing a single genotype to potentially dominate the population. By spatially separating individual mutants, growth-based competition is eliminated, preserving the diversity of mutants of interest and enabling their selection. Such spatial separation can be achieved by separating colony-forming units on solid medium. However, this approach requires large volumes of culture medium and laboratory consumables, as well as quantities of antimetabolites. Many antimetabolites are expensive and toxic to humans. Miniaturisation of screening in microtiter plates, combined with the use of liquid-handling robotics, improves screening throughput but is still insufficient to cover the size of mutant pools (Zeng et al. 2020).

Water-in-oil emulsions of agarose microbeads contain a large number of individual picolitre-sized cultivation compartments (~ 10^7 microbeads) in just 0.3 mL of medium (van Tatenhove-Pel et al. 2020; Zeng et al. 2020; Zhou & Alper 2019). This study aims to investigate the potential of this multi-parallel cultivation method to select for antimetabolite-resistant mutants from large cell populations while minimising total culture volume. When each microbead, containing medium with an antimetabolite, is inoculated with a single cell, only beads inoculated with an antimetabolite-resistant cell are expected to show growth. Microbeads exhibiting growth can then be selected by (auto)fluorescence-activated microbead-sorting, to enrich for mutants with antimetabolite resistance in a label-free manner—unlike most existing microdroplet-based selection methods, which rely on genetically engineered fluorescent markers (van Tatenhove-Pel et al. 2020). The combination of microbead cultivation with microbead-sorting should allow for label-free screening of millions of mutants while using minimal amounts of expensive and toxic antimetabolites. Additionally, since beads are inoculated with single cells, genetic diversity in the mutant population can be fully captured. To test this approach, we developed a microbead-based, label-free method to enrich for antimetabolite-resistant *S. cerevisiae* strains from a population of sensitive *S. cerevisiae* strains. We validated the method by enriching G418 (geneticin) and 2-deoxyglucose (glucose antimetabolite) resistant *S. cerevisiae* strains.

Materials and methods

Strains, media, and cultivation

Plasmids and *Saccharomyces cerevisiae* strains used in this study are shown in Table S2 (supplementary Sect. 2) and Table 1, respectively. Plasmids were propagated in *Escherichia coli* XL1-Blue cells (Agilent, Santa Clara, CA, USA). For long-term storage at − 80 °C, glycerol was added to late-exponential-phase *S. cerevisiae* cultures and overnight *E. coli* cultures to obtain final concentrations of 30% (v/v) and 25% (v/v), respectively.

The *E. coli* cultures were grown at 37 °C in LB medium supplemented with 100 µg/L ampicillin for maintenance of

Table 1 *S. cerevisiae* strains used in this study

S. cerevisiae strain	Genotype	Reference
CEN.PK113-7D	*MATa URA3 TRP1 LEU2 HIS3*	
IMX2600	*MATa URA3 TRP1 LEU2 HIS3 can1Δ::*cas9-*natNT2*	van den Broek et al. 2024
IMI575	*MATa URA3 TRP1 LEU2 HIS3 can1Δ::*cas9-*natNT2 X-2::*p*AgTEF-kanMX*-t*AgTEF*	This study
IMX2871	*MATa URA3 TRP1 LEU2 HIS3 can1Δ::*cas9-*natNT2 X-2::* p*TDH3*-ymNeongreen-t*ENO2*	This study
IMX2748	*MATa URA3 TRP1 LEU2 HIS3 can1Δ::*cas9-*natNT2 hxk2Δ::SynPAM*	This study
IMI602	*MATa URA3 TRP1 LEU2 HIS3 can1Δ::*cas9-*natNT2 hxk2Δ::SynPAM X-2::*p*AgTEF-kanMX*-t*AgTEF*	This study

plasmids. The *S. cerevisiae* strains were grown at 30 °C in 500 mL or 100 mL round-bottom flasks with 100 mL or 20 mL medium, respectively at 200 rpm in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) and incubated for 18 h. The *S. cerevisiae* strains were grown either in yeast extract peptone medium (YP, 10 g/L Bacto yeast extract and 20 g/L Bacto peptone), two-fold concentrated yeast extract and peptone medium (HCYP, 20 g/L Bacto yeast extract and 40 g/L Bacto peptone) or in synthetic medium suitable for cultivation of cultures at high cell concentrations (HCSM). The HCSM was prepared as described by Castrillo et al. (1996) but without antifoam and substituting $(NH_4)_2SO_4$ with 9 g/L urea. Pre-cultures were grown in YP supplemented with 20 g/L glucose. The HCYP and HCSM were supplemented with 80 g/L glucose and 80 g/L sucrose, respectively. When *kanMX* was used for selection, 50 to 600 µg/mL of sterilised G418 (geneticin) was added to the HCYP medium. Although chemically defined medium (HCSM) is preferred, it was not used for G418-based selection as the growth of the G418 sensitive strains was not effectively inhibited in this medium. The inhibition was potentially caused by the high concentration of potassium and magnesium containing salts in the medium. When 2-deoxyglucose was used for selection, 0.25 to 8 mg/mL of sterilised 2-deoxyglucose was added to the HCSM medium. Solid medium plates contained 2% (w/v) agar.

Molecular biology techniques and strain construction

Plasmids were isolated from *E. coli* using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Genomic *S. cerevisiae* DNA was isolated as previously described by Lõoke et al. (2011). DNA amplifications were performed using Phusion High Fidelity DNA polymerase (Termo Fisher Scientifc, Waltham, MA, USA) for cloning, and DreamTaq PCR Mastermix (Termo Fisher Scientifc) for diagnostic PCRs. For isolation of DNA fragments from the PCR reaction mixture, GeneJET PCR Purifcation Kit (Termo Fisher Scientifc) was used. DNA fragments were separated and visualised on a 1% agarose TAE gel by 30-min electrophoresis at 100 V. The DNA fragments excised from the gel were purified using the Zymoclean Gel DNA recovery Kit (Baseclear). Gibson assemblies of plasmids were performed using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA) and the resulting plasmids were propagated in XL1-Blue chemically competent *E. coli* cells. Transformations of *S. cerevisiae* strains were performed using LiAC/ssDNA/PEG, as previously described by Gietz and Woods (2002). After the transformations, gRNA plasmids were removed as described by Mans et al. (2015) and the single colonies were re-streaked at least three consecutive times to ensure isogenic single cell lines. Genomic insertions were confirmed by performing diagnostic PCRs on the isolated genomic DNA.

Table 1 and Table S2 show the strains and plasmids, respectively, used in this study. In IMX2600, *HXK2* was deleted by introducing pUDR371, along with a double-stranded DNA repair fragment obtained by annealing primers 18,833 and 18,834 (primers used in the study are listed in supplementary Sect. 1), resulting in strain IMX2748. A *kanMX* integration cassette was amplified from plasmid pUG6 using primers 20,226 and 20,227. The *kanMX* integration cassette, along with the plasmid pUDR376, was transformed into IMX2600 and IMX2748, resulting in IMI575 and IMI602, respectively. The pGGKd016 was constructed by assembly of part plasmids pYTK002, pYTK047, pYTK072, pYTK074, pYTK083, and pYTK081 using the Golden Gate reaction protocol described by Lee et al. (2015). The plasmid pUDE1242 was created by Gibson assembly of three fragments that were PCR amplified from pUDE1111, pUDC329, and pGGKd016 with primer pairs 17,634 and 19,385, 12,382 and 14,776, and 14,777 and 17,008, respectively. A ymNeongreen integration cassette was amplified from pUDE1242 using primers 19,247 and 19,248. The ymNeongreen integration cassette, along with the plasmid pUDR538, was transformed into IMX2600, resulting in IMX2871. The ymNeongreen marker is codon-optimised for yeast and is characterised as one of the best performing fluorescent protein markers in *S. cerevisiae*

(based on in vivo brightness, photostability) (Botman et al. 2019).

Analytical methods

Optical density (OD) was measured at 660 nm using a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Cell concentration was measured using a BD Accuri™ C6 Plus flow cytometry (BD Biosciences, San Jose, CA, USA). Size and volume distributions of the agarose beads in oil were determined by imaging with a Zeiss Axio Imager Z1 (Carl Zeiss AG, Jena, Germany) and an AxioCam HRm Rev3 detector (60 N-C 1″ × 1.0) (Carl Zeiss AG) using the lateral magnification objective × 20. Details on the estimation of the average bead size are described in Sect. 3 of the supplementary information.

Preparation of microbeads

Agarose microbeads in oil were prepared as described by van Tatenhove-Pel et al. (2021). The emulsions were prepared with a water phase and an oil phase (Novec HFE 7500 fluorinated oil, 3 M, Maplewood, MN, USA) containing 0.2% PicoSurf 1 surfactant (Sphere Fluidics, Cambridge, UK). The water phase contained HCYP medium with 8% (wt/v) glucose or HCSM with 8% (wt/v) sucrose, and 1% (wt/v) agarose with ultra-low gelling temperature (Type IX-A, A2576, Sigma-Aldrich, Saint Louis, MO, USA). Where indicated, G418 or 2-deoxyglucose and yeast cells were also added to the water phase.

Cells from pre-cultures were harvested during exponential growth (at an OD_{660} between 4 and 10). The cells were centrifuged (5 min 3000 × *g*), washed with sterile demineralised water, and after which the cell concentration (cells/mL) was measured. 2.7×10^6 cells were diluted in 300 µL of HCSM with 8% (wt/v) sucrose or HCYP with 8% (wt/v) glucose for a λ value of 0.2 (supplementary Sect. 4). At this λ value, 90% of inoculated microbeads (excluding empty microbeads) contain one cell (supplementary Sect. 4). Two times the required number of cells to inoculate at a λ value of 0.2 were added to account for loss of cell viability during preparation of the microbeads. To prepare the emulsions, 300 µL of the water phase was mixed with 700 µL of the oil phase with the surfactant using a T10 basic ULTRA TURRAX homogeniser with an S10N-5G dispersing element (IKA, Staufen, Germany) at speed 3 for 5 min. Two-millilitre round-bottom tubes (Eppendorf, Hamburg, Germany) were used to prepare and incubate the emulsions. Emulsions were prepared at room temperature. Microbeads prepared with HCYP with 8% (wt/v) glucose medium had an average volume of 52.12 pL (supplementary Sect. 3). Microbeads prepared with HCSM with 8% (wt/v) glucose medium had an average volume of 47.40 pL (supplementary Sect. 3). Emulsions were incubated on a HulaMixer® Sample Mixer (Thermo Fisher Scientific, Waltham, MA, USA) at 30 °C while rotating at 1-rpm orbital rotation for 40 h. After incubation, microbeads were separated from the oil phase by adding 1.2 mL phosphate-buffered saline (PBS) and 1.5 mL perfluorooctanol (PFO, Alfa Aesar, Ward Hill, MA, USA) to 1 mL of the emulsion and gently mixing. Microbeads partitioned to the water phase (i.e. PBS), which was carefully recovered with a pipette.

Sorting of microbeads

Before flow cytometry, microbeads larger than 40 µm were removed by filtering over a 40-µm mesh filter (pluriStrainer, PET-mesh). Microbeads smaller than 40 µm were subsequently measured. Forward scatter, side scatter, and green fluorescence signal were measured using the 488-nm laser on a BD FACSAria™ II SORP Cell Sorter (BD Biosciences, San Jose, CA, USA), operated with FACSFlow™ software (BD Biosciences) and equipped with a 130-µm nozzle. The green fluorescence signal was detected through a 545/28-nm or a 530/30-nm bandpass filter. Between 96 and 384 events in the gates of interest were sorted with 4-way purity precision on agar plates with YP + 2 wt% glucose. FlowJo™ v10.10 software (BD Life Sciences) was used for data analysis. The applied gating strategy is described in detail in the supplementary Sect. 5.

Determining relative proportions of strain types

Relative abundances of strains in the inoculum and after microbead sorting were determined to assess enrichment. Relative abundance of *S. cerevisiae* strains IMX2871 and IMI575 (fluorescent and non-fluorescent strains) in inocula for emulsion cultures was determined by measurement of fluorescence using flow cytometry (10^5 events recorded). To determine the fraction of different strains after sorting microbeads in phosphate buffer saline, microbead samples were first vortexed, and then the resulting suspension was plated on YP with 2% glucose (wt/v) solid medium. After incubation of the plates at 30 °C for at least 18 h, relative abundances of the two strains were determined by counting the fluorescent and non-fluorescent colonies after illuminating plates with Safe Imager 2.0 Blue-light Transilluminator (Invitrogen, MA, USA).

To determine the relative proportions of non-fluorescently labelled resistant and non-resistant strains before inoculating microbeads, we plated the mixtures on YP with 2% (wt/v) glucose solid medium plates with G418 as a selection marker (the 2-deoxyglucose resistant strain is also engineered with resistance to G418). Depending on the target initial fractions (ranging from 1 in 10^2 to 1 in 10^4), between 10^4 and 10^6 cells from each mixture were plated, respectively. Microbeads

were inoculated with a mix of the strains, and the beads with growth were sorted on YP with 2% (wt/v) glucose solid medium plates. All colonies from the YP with 2% (wt/v) glucose solid medium plates were scored for growth on YP with 2% (wt/v) glucose solid medium plates with G418 as a selection marker.

Results

Label-free identification of a resistant *S. cerevisiae* strain

To identify microbeads with the growth of an unlabelled resistant strain in the presence of an inhibitor molecule, microbeads were inoculated with G418-sensitive CEN.PK113-7D and G418-resistant IMI575 *Saccharomyces cerevisiae* strains. The G418 is an aminoglycoside antibiotic that inhibits protein synthesis and is commonly used as a selection marker in *S. cerevisiae* (Wach et al. 1994).

Flow cytometry analysis of empty microbeads showed a distribution of side-scatter and fluorescence signals, which is consistent with their polydispersity. Microbeads were inoculated at a λ value of 0.2 (supplementary Sect. 4) and incubated for 40 h in the presence of the antibiotic G418. After growth, flow cytometry analysis showed that microbeads inoculated with cells contained an additional population compared to empty microbeads (Fig. 1a, c, and d). This population was identified as single cells by comparing to a control sample (Fig. 1b). The single cell population likely arises from damaged microbeads. For microbeads inoculated with the G418-resistant strain IMI575, an additional population with both higher fluorescence and side scatter signal was detected (Fig. 1d). This population was identified as microbeads with cell growth, as increased autofluorescence is expected with growth and was only observed for the microbeads inoculated with the resistant strain (Fig. 1d). Increase in the side scatter signal is consistent with greater internal complexity of the microbeads resulting from cell growth.

These results show that the growth of an unlabelled resistant strain in microbeads can be detected using autofluorescence and/or side scatter as a growth indicator. A detailed overview of the general gating strategy employed is described in the supplementary Sect. 5.

Enrichment of *S. cerevisiae* cells expressing a fluorescent protein using microbead sorting

Validation of the proposed enrichment method for a specific, low-abundance yeast strain, was performed using mixtures of the ymNeongreen-expressing, fluorescent strain IMX2871

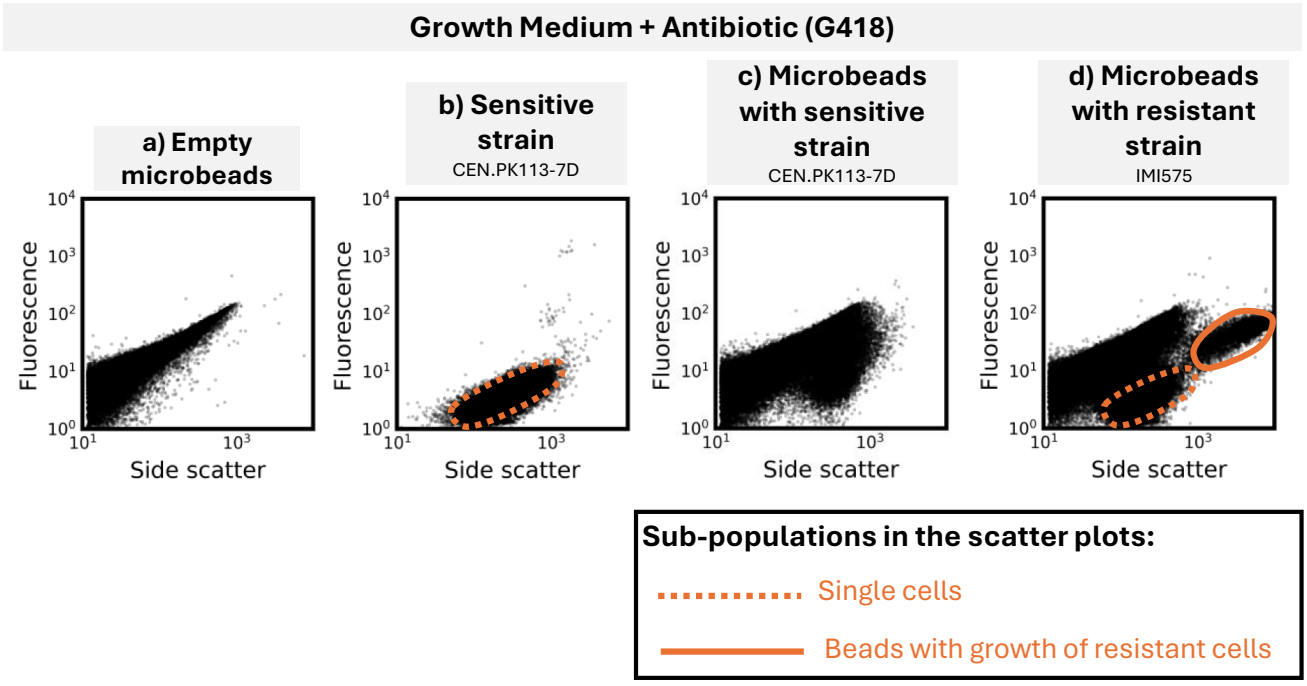

Fig. 1 Identification of flow cytometry populations corresponding to **a** empty microbeads, **b** G418-sensitive *S. cerevisiae* strain CEN.PK113-7D single cells, **c** microbeads inoculated with the G418-sensitive strain, and **d** microbeads inoculated with a G418-resistant *S. cerevisiae* strain IMI575. Microbeads were prepared with medium containing G418, inoculated at a λ value of 0.2, and incubated for 40 h. Additional gating information is provided in supplementary Sect. 5

and the *kanMX*-expressing, G418-resistant *S. cerevisiae* strain IMI575.

To minimise the number of microbeads inoculated with more than one cell, while maintaining a sufficient number of inoculated microbeads, microbeads were inoculated at a λ-value of 0.2 (supplementary Sect. 4). As anticipated, after growth, flow cytometry showed a higher fluorescence of microbeads inoculated with the ymNeongreen-expressing *S. cerevisiae* strain IMX2871 than of microbeads inoculated with the *kanMX*-expressing reference strain IMI575 (supplementary Sect. 5). The fluorescence signal from microbeads inoculated with the reference strain was attributed to the autofluorescence of yeast cells and the agarose matrix used to generate the microbeads (Siano and Mutharasan 1989; Podrazký et al. 2003; Maslanka et al. 2018).

Based on the fluorescence of the two strains, a FACS gate was defined to specifically sort the ymNeongreen-expressing strain IMX2871. After sorting, the relative abundances of the two strains were measured using flow cytometry. In three independent single-round sorting experiments, strain IMX2871 was enriched from a starting abundance of 0.096% (1 in 1000) to a final fraction of 78% ± 7%, which represents an 800-fold enrichment.

Even though a fluorescently labelled yeast strain was used for validation of a label-free enrichment method, these results serve as proof of concept that FACS-based microbead can be used to strongly enrich a specific yeast strain from a mixed culture in which it occurs at a low abundance.

Enrichment of a G418-resistant *S. cerevisiae* strain by using microbead sorting

A next set of experiments aimed to investigate whether the proposed approach in this study can be used to enrich microbeads containing a low-abundance inhibitor-tolerant strain from a mixed population, without using a heterologous expressed fluorescent protein. To this end, experiments were designed to select the *kanMX*-expressing, G418-resistant *S. cerevisiae* strain IMI575 from mixtures with the G418-sensitive strain CEN.PK113-7D.

Concentrations of G418 required to inhibit growth in suspension and microbead cultures were determined (Fig. 2). Suspension cultures on HCYP medium with 8% (wt/v) glucose in shake flasks were inoculated at a cell concentration equivalent to one cell per microbead (3×10^7 cells/mL). The high sugar concentration was used to ensure sufficient growth of the strains within the microbeads to be able to distinguish between microbeads with and without growth. At G418 concentrations between 200 and 600 µg/mL, cell concentrations in suspension cultures measured after incubation remained unchanged compared to the cell concentration measured immediately after inoculation (Fig. 2a).

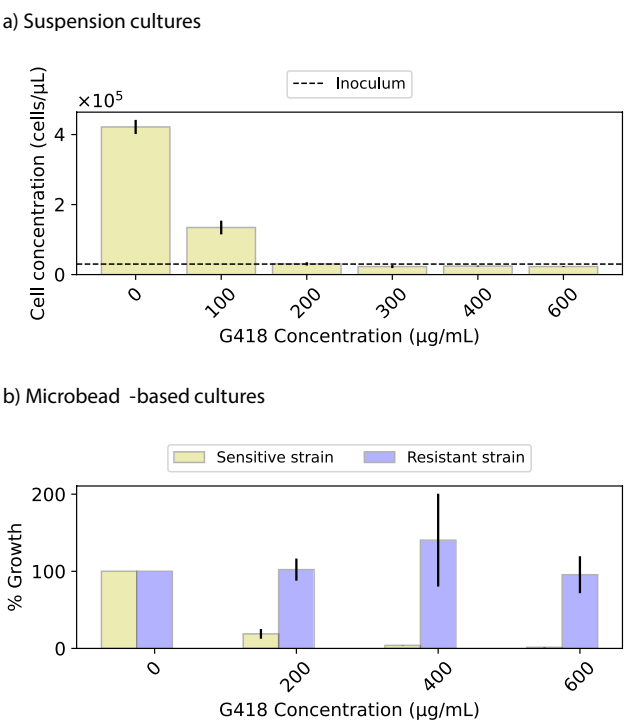

Fig. 2 Analysis of growth inhibition of suspension and microbead cultures at different concentrations of G418. **a** Cell concentrations (cells/µL) at different G418 concentrations (µg/mL) measured in suspension cultures of the G418-sensitive *S. cerevisiae* strain IMX2871. The dashed line indicates the cell concentration of the inoculum, which corresponds to one cell per microbead. **b** Percentage of microbeads with growth in the presence of G418 normalised to the proportion of microbeads with growth in the absence of G418. Microbeads were inoculated at λ value of 0.2 with the G418-sensitive strain IMX2871 (yellow bars) or the G418-resistant strain IMI575 (purple bars) and were incubated for 40 h

The same concentration range of G418 that was evaluated in suspension cultures was tested in microbead cultures inoculated at λ value of 0.2. To quantify inhibition by G418, using flow cytometry, we first identified the percentage of microbeads showing growth (Fig. 1 and gated as shown in supplementary Sect. 5). We then normalised the fraction of microbeads showing growth in the presence of G418 to the fraction observed in the absence of the antibiotic (Fig. 2b). Using the same 200 to 600 µg/mL concentration range of G418 used in the suspension cultures, despite variation in the data, clear growth of the resistant strain IMI575 in microbeads was observed (Fig. 2b). In contrast to suspension cultures, microbead cultures of the G418-sensitive strain IMX2871 showed growth at G418 concentrations of 200 and 400 µg/mL. However, also in microbeads, a G418 concentration of 600 µg/mL sufficed to prevent growth of the sensitive strain (Fig. 2b).

We subsequently assessed whether exposure of microbead cultures of G418-sensitive *S. cerevisiae* to 600 µg/mL killed or merely inhibited growth. To this end, microbead cultures

with and without G418 were inoculated with the sensitive strain CEN.PK113-7D. After 40-h incubation, sorted events corresponding to single cells and microbeads inoculated with cells (gated as shown in supplementary Sect. 5) were placed on non-selective plates to check for growth. Of the sorted events from cultures grown in the absence of G418, 71% ± 4% yielded colony-forming units, while none of the sorted events from G418-supplemented cultures showed any colony-forming units (supplementary Sect. 6). These results indicate that only G418-resistant strains survive G418 exposure in microbeads and subsequent sorting.

To evaluate whether microbeads containing a G418-resistant strain can be selected by FACS when they only represent a small fraction of the total fraction of inoculated beads, mixtures of the resistant strain IMI575 and the sensitive strain CEN.PK113-7D were prepared at ratios of $1{:}10^2$, $1{:}10^3$, and $1{:}10^4$ (Fig. 3). Microbeads were then inoculated with these mixtures at a λ value of 0.2. After incubation for 40 h, microbeads with the highest autofluorescence and side scatter signal were selected. A substantial enrichment of the G418-resistant strain IMI575 was observed for all initial strain ratios. Four separate replicate sort experiments were performed with initial strain mixtures that contained 0.72% ± 0.03% resistant cells. In each replicate sort experiment, microbeads with growth were sorted. All the resulting colonies were resistant cells (100% ± 0%) (Fig. 3). Similarly, in four separate sort experiments with mixtures containing 0.059% ± 0.025% resistant cells, 98.60% ± 2.42% of the obtained colonies were resistant cells (Fig. 3). In the most extreme case, with an initial proportion of the resistant strain of 0.0063 ± 0.0026%, in three separate sort experiments, all the resulting colonies were resistant cells (100% ± 0%) (Fig. 3).

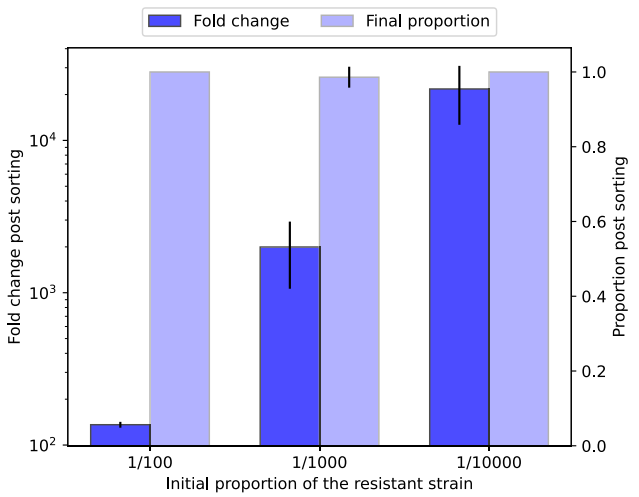

Fig. 3 Fold-change and proportion of the G418-resistant *S. cerevisiae* strain IMI575 before and after microbead sorting. The target ratios for the mixtures of the resistant strain IMI575 and the sensitive strain CEN.PK113-7D ($1{:}10^2$, $1{:}10^3$, and $1{:}10^4$) correspond to average measured ratios of 1:139 ± 6 (two mixtures), 1:2037 ± 846 (two mixtures), and 1:19,135 ± 7879 (two mixtures). Microbeads were inoculated at λ value of 0.2 with the strain mixtures and incubated in medium containing G418. After incubation for 40 h, microbeads with growth were sorted. Each bar represents the average from four sorting experiments ($1{:}10^2$ and $1{:}10^3$) or three experiments ($1{:}10^4$). The relative abundances of the resistant and sensitive strains were quantified before and after sorting

The results demonstrate that microbeads inoculated with a G418-resistant strain can be enriched by 16,000-fold in a single round of sorting from a mixture with a sensitive strain in which it occurs at very low frequencies ($1{:}10^4$). The earlier viability assessment shows that G418-sensitive strains do not recover after exposure to G418 under these conditions, which explains the high enrichment observed after sorting, even in the presence of known sorting inefficiencies.

Enrichment of a 2-deoxyglucose-resistant *S. cerevisiae* strain by using microbead sorting

To assess whether the proposed approach for enriching specific, low-abundance phenotypes from microbead cultures can be applied to select for cells resistant to a non-lethal antimetabolite, 2-deoxyglucose was used as a model antimetabolite. 2-Deoxyglucose is a glucose analogue that lacks a hydroxyl group at the second carbon, mimics glucose, and thereby represses the utilisation of alternative carbon sources (Laussel and Léon 2020). 2-Deoxyglucose is commonly used to select for yeast mutants that, due to alleviation of glucose catabolite repression, can convert other carbon sources in the presence of glucose (Neigeborn and Carlson 1987; McCartney et al. 2014a; Soncini et al. 2020).

To determine the concentration of 2-deoxyglucose to inhibit the growth of suspension cultures on sucrose (Fig. 4a), batch cultures were inoculated with the sensitive *S. cerevisiae* strain CEN.PK113-7D or the *hxk2*Δ-resistant strain IMX2748. The resistant strain lacks *Hxk2*, as this deletion has been shown to confer the strain resistance to 2-deoxyglucose in the presence of sucrose. There are several hypotheses for reduced toxicity due to the deletion of Hxk2: reduced phosphorylation to 2-deoxyglucose-6-phosphate (McCartney et al. 2014b; Soncini et al. 2020; Schmidt and O'Donnell 2021), alleviation of repression of sucrose invertase (Suc2) (Neigeborn and Carlson 1987; Soncini et al. 2020), and/or possible detoxification of 2-deoxyglucose-6-phosphate via the expression of 2-deoxyglucose-6-phosphatases (Laussel and Léon 2020). Microbead cultures were inoculated at a cell concentration of 3×10^7 cells/mL (corresponding to one cell per microbead). At 2-deoxyglucose concentrations of 0.5 g/L or higher, for CEN.PK113-7D, no increase in the cell number was observed after inoculation (Fig. 4a and b). In similar experiments with the *hxk2*Δ strain IMX2748, clear growth was observed at all tested 2-deoxyglucose concentrations, although final cell concentrations

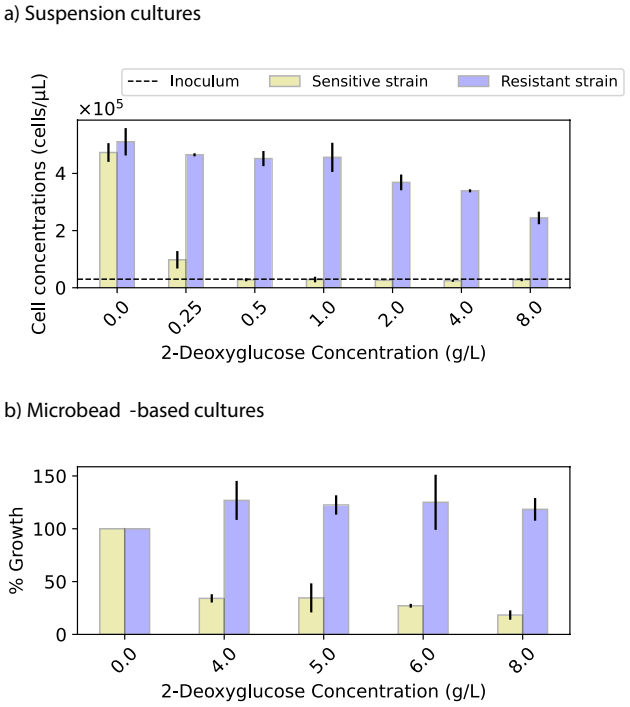

Fig. 4 Analysis of growth inhibition of suspension and microbead cultures at different concentrations of 2-deoxyglucose. **a** Cell concentrations (cells/µL) at different 2-deoxyglucose concentrations (g/L) measured in suspension cultures of the 2-deoxyglucose-sensitive strain CEN.PK113-7D (yellow bars) and the 2-deoxyglucose-resistant strain IMX2748 (purple bars). The dashed line indicates the cell concentration of the inoculum, which corresponds to one cell per microbead. **b** Percentage of microbeads with growth in the presence of 2-deoxyglucose normalised to the proportion of microbeads with growth in the absence of 2-deoxyglucose. Microbeads were inoculated at $\lambda = 0.2$ with the 2-deoxyglucose-sensitive strain CEN.PK113-7D (yellow bars) or 2-deoxyglucose-resistant strain IMI602 (purple bars) and incubated for 40 h

showed a negative correlation with 2-deoxyglucose concentration (Fig. 4b). This correlation is likely due to the toxicity of 2-deoxyglucose, which is still taken up by the cell and, due to phosphorylation by hexose kinases, causes accumulation of non-metabolisable intermediates and ATP depletion (Laussel and Léon 2020; Luzia et al. 2023).

To test growth inhibition by 2-deoxyglucose in sucrose-grown microbead cultures, they were inoculated at a λ value of 0.2 with the sensitive *S. cerevisiae* strain CEN.PK113-7D or the *hxk2*Δ-resistant strain IMI602. Growth at different 2-deoxyglucose concentrations by flow cytometry was based on autofluorescence and side scatter signal (Fig. 1). To quantify inhibition by 2-deoxyglucose, using flow cytometry, we first estimated the percentage of microbeads showing growth (gated as shown in supplementary Sect. 5). We then normalised the proportion of microbeads showing growth in the presence of 2-deoxyglucose to the proportion observed in the absence of the antimetabolite (Fig. 4b). Based on these experiments, a 2-deoxyglucose concentration of 8 g/L was chosen for further studies in microbead cultures. As observed for G418, microbead cultures were less sensitive to 2-deoxyglucose than suspension cultures (Fig. 4).

To assess the effect of 2-deoxyglucose on a sensitive strain, microbeads were inoculated with the sensitive strain CEN.PK113-7D in the absence and presence of the inhibitor at 8 g/L. After 40 h of incubation, FACS events identified as single cells and microbeads inoculated with cells (gated as shown in supplementary Sect. 5) were plated on non-selective medium plates. Of the events sorted from microbead cultures grown in the absence of 2-deoxyglucose, $84 \pm 11\%$ showed growth as colony-forming units on plates. A much lower fraction of colony-forming units ($9 \pm 6\%$) was observed for events sorted from microbead cultures grown in the presence of 2-deoxyglucose (supplementary Sect. 6). These results show that only a small percentage of 2-deoxyglucose-sensitive strains can form colonies after incubation with 8 g/L 2-deoxyglucose in microbeads, followed by microbead-sorting.

To test the feasibility of sorting microbead populations for low-abundance microbeads containing 2-deoxyglucose-inhibition-resistant cells, the resistant strain IMI602 and the sensitive strain CEN.PK113-7D were mixed at ratios of $1{:}10^3$ and $1{:}10^4$. Microbeads were inoculated with these mixtures at λ value of 0.2 and, after incubation for 40 h, beads with high autofluorescence and side scatter signals were selected. For both initial strain ratios, this procedure yielded an enrichment of the 2-deoxyglucose-resistant strain IMI602. IMI602 was also engineered to be resistant to G418 to enable determination of relative abundances (pre- and post-sorting) by screening for resistance to the antibiotic. In each separate replicate sort experiment, microbeads with growth (gated as shown in the supplementary Sect. 5) were sorted. In mixtures initially containing $0.12\% \pm 0.0076\%$ of strain IMI602, from six separate replicate sort experiments, the resistant strain was enriched to $7.15\% \pm 3.12\%$. Similarly, for mixtures containing $0.0085\% \pm 0.00029\%$, from three separate replicate sort experiments, the resistant strain was enriched to $5.70\% \pm 3.77\%$ (Fig. 5).

The results show that, although the enrichment of the resistant strain did not reach 100%, an approximately 600-fold enrichment of an antimetabolite-resistant strain was achieved in a single round of sorting, starting from a strain mixture in which it represented approximately $1{:}10^4$ of the population. The earlier viability assessment showed that 2-deoxyglucose-sensitive strains can recover after exposure to 2-deoxyglucose under these conditions. This, together with potential (co-)sorting of populations outside the gated population to be sorted could explain the presence of sensitive strains.

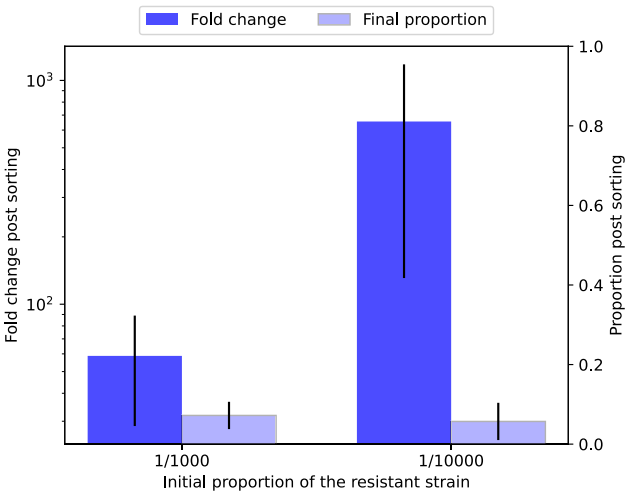

Fig. 5 Fold-change and proportion of the 2-deoxyglucose-resistant *S. cerevisiae* strain IMI602 before and after microbead sorting. The target ratios for the mixtures of the resistant strain IMI575 and the sensitive strain CEN.PK113-7D ($1:10^3$ and $1:10^4$) correspond to average measured ratios of $1:806 \pm 50$ (two mixtures) and $1:11{,}755 \pm 409$ (two mixtures). Microbeads were inoculated at $\lambda = 0.2$ with the strain mixtures and incubated in medium containing 2-deoxyglucose. After incubation for 40 h, microbeads with growth were sorted. Each bar represents the average from six sorting experiments ($1:10^3$) or three experiments ($1:10^4$). The relative abundances of the resistant and sensitive strains were quantified before and after sorting

Discussion

The results presented in this study demonstrate cultivation in agarose-bead-in-oil emulsions combined with label-free high-throughput sorting, enabling fast and efficient selection of antimetabolite-resistant *S. cerevisiae* strains from large cell populations. Use of microbeads minimised costs by enabling the reduction of the water phase of the selection cultures to 0.3 mL. A 600 to 16,000-fold enrichment of microbeads with resistant cells in the subsequent sorting step minimised the medium requirement for further selection on solid media or in microtiter plates. This approach has the potential to strongly reduce the amounts of antimetabolites and other laboratory consumables needed in screening campaigns. Finding a non-toxic replacement for the fluorinated oil used in the cultivation step is a relevant challenge in attempts to further reduce the environmental impact of this approach. By using autofluorescence and side scatter signals as an indicator for growth, sorting of microbeads with resistant cells could be performed without expressing a heterologous fluorescent protein. In cases where autofluorescence signals from the microorganism of interest are too weak to use as an indicator for selection of microbeads with growth, the use of fluorescent markers that are activated by cellular metabolism (Maurice and Turnbaugh 2013; Kehe et al. 2019) may offer an interesting alternative.

Although complete enrichment of the 2-deoxyglucose-resistant strain was not achieved, a 600-fold enrichment was observed. In the most challenging case, with an initial resistant fraction of $0.0085\% \pm 0.00029\%$, the resistant strain was enriched to $5.70\% \pm 3.77\%$. This pre-enrichment demonstrates that applying the proposed approach to a mutant pool can drastically reduce the screening effort from ~ 10^5 mutants to only a few hundred. Such a substantial reduction enables feasible identification of mutants of interest, for example, using 96-well plate-based assays. Subsequent rounds of sorting are expected to further increase enrichment. Altogether, the proof of concept using the antimetabolite 2-deoxyglucose showcases significant enrichment of the strain of interest, despite a non-lethal effect of the antimetabolite.

Use of water-in-oil emulsions implies that partitioning of hydrophobic antimetabolites into the oil phase may complicate the adoption of this method. If this problem cannot be addressed by increasing the amount of antimetabolite added to the assays, without considerably increasing the costs of screening, it may be attempted to increase their concentration in the water phase by measures such as adding bovine serum albumin, adjusting surfactant concentrations, or chemically modifying the antimetabolite (Gruner et al. 2016; Scheler et al. 2016; Skhiri et al. 2012). Growth in emulsions may also be influenced by factors such as oxygen supply and pH. However, these variables would not affect the enrichment outcome, provided the cells undergo sufficient number of doublings within the microbeads to allow reliable distinction between growth and no growth.

Minimum inhibitory concentrations (MICs) of both G418 and 2-deoxyglucose in microbead cultures were higher than in suspension cultures. Context dependency of MIC values does by no means preclude the use of microbead-based selection, but was not anticipated in this study since, based on their partition coefficients, G418 and 2-deoxyglucose (Log$P$ values are -1.37 and -3.07, respectively) should remain in the aqueous phase (ChemSpider 2025). Instead, the different MICs in microbead and suspension cultures may reflect different physiology of microbead-grown cultures, partial inactivation or degradation of inhibitors during emulsion preparation, and/or binding to agarose.

In cases where an inhibitor fully blocks re-growth of sensitive cells, as observed in this study for selection with the antibiotic G418, using low-volume suspension cultures, selection of mutant mixtures in the presence of such an antimetabolite might suffice. However, selection in suspension cultures could result in loss of genetic diversity, as the fastest-growing surviving mutants will dominate the population. Eliminating this competition by spatial segregation in microbeads allows for more representative sampling of the genetic diversity of mutant pools.

2-Deoxyglucose-resistant yeast cells were selected by growing them on sucrose, a substrate whose utilisation by *S. cerevisiae* is repressed by glucose (Raamsdonk et al. 2001; Kayikci and Nielsen 2015). In *S. cerevisiae*, metabolism of sucrose is initiated by hydrolysis to fructose and glucose, a reaction that predominantly occurs extracellularly (Basso et al. 2011). In the microbead emulsions, the fructose and glucose are retained in the water phase, which implies that they are only accessible for the clonal culture inside the microbead. This privatisation of resources differs from the situation in suspension cultures and solid media, where all cells share extracellular substrates as well as extracellular enzymes. The simple, label-free selection and screening method described in this study may therefore be applicable for the selection of microorganisms that efficiently hydrolyse extracellular substrates. Such an application may, for example, be relevant for the improvement of yeast strains for consolidated bioprocessing (i.e. simultaneous extracellular hydrolysis and fermentation of polymeric or oligomeric saccharides; Gao et al. 2019).

In setting up the microbead cultivation and sorting strategy, we used the antibiotic G418 as a model inhibitor. Rapid detection and identification of effective antibiotic regimes of clinical isolates are critical for enabling timely treatment of microbial infections. Conventional assays often require long incubation times to reach detectable cell concentrations (Boedicker et al. 2008; Agnihotri et al. 2025). Boedicker et al. (2008) and Agnihotri et al. (2025) addressed this problem by using small-volume confinements to increase the local cell concentration. However, their strategies required specialised microfluidic devices and imaging. The simpler microbead-emulsion method presented here may offer a simple high-throughput alternative that, as long as a flow cytometer or FACS is available, can easily be implemented in clinical laboratories.

In conclusion, the high-throughput microbead-based selection method offers a label-free, cost-reducing alternative to conventional methods for enriching antimetabolite-resistant *S. cerevisiae* strains. The method was validated by achieving a 600-fold enrichment of a 2-deoxyglucose (glucose analogue) resistant *S. cerevisiae* strain and a 16,000-fold enrichment of a G418 (antibiotic)-resistant *S. cerevisiae* strain. Beyond this application, the simple, label-free and high-throughput design may also be applicable for selecting mutants with improved extracellular product hydrolysis substrates and rapid identification of effective antibiotic regimes with clinical isolates.

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Author contributions JTP, RJvT, and TW conceptualised the work. JTP, RJvT, and SBG designed experiments. SBG wrote the original draft of the manuscript, which was reviewed and edited by JTP, RJvT, and TW. SBG performed the experiments and conducted data analysis. All authors read and approved the final manuscript.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval This declaration is not applicable.

Competing interests The authors declare no competing interests.

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