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✉ For correspondence:

idris.kempf@eng.ox.ac.uk

harrison.steel@eng.ox.ac.uk

† These authors contributed equally to this work.

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Microscopic PhotoSelection (MiPS) of single cells in mother machine microfluidic devices

Jessica James[†], Idris Kempf[†] ✉, Kirill Sechkar, Jingyu Wang, Gabriel Abrahams, Sebastian Towers, Harrison Steel ✉

Department of Engineering Science, University of Oxford, Oxford, United Kingdom

eLife Assessment

The manuscript presents a **compelling** application of the microfluidic mother machine as a "selection" machine. While individual elements of the work have existed for many years across various research communities, the simplicity and elegance of this particular application should make it readily implementable and therefore a **useful** contribution. The proposed plans for revision are sound, particularly in light of the senior author's stated logistical constraints.

[Editors' note: this paper was reviewed by *Review Commons* [✉](#).]

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Abstract

Techniques for selecting or sorting single cells within large populations of genetic variants are central to synthetic biology and biotechnology. Widely-used methods such as Fluorescence Activated Cell Sorting (FACS) enable rapid processing of large libraries, but are restricted to low-dimensional measurements taken at a single time point. As a result, sorting based on dynamic or multi-trait phenotypes—such as transient properties and properties that occur on fast timescales or in response to dynamic actuating signals—remains fundamentally challenging. Here we introduce Microscopic PhotoSelection (MiPS) which employs an automated robotic platform for single-cell selection based on multiple dynamic criteria, directly on microfluidic mother machine devices. The system couples long-term single-cell imaging with real-time analysis and selective optical targeting, allowing fully automated enrichment using high-intensity UV light or alternative wavelengths with the addition of photosensitisers. By targeting many cells in parallel, our platform overcomes throughput limitations of existing microfluidic-based selection technologies such as optical tweezers or droplet-based methods and provides a direct approach to select cells based on time-resolved, multi-trait phenotypes. We demonstrate the ability to perform *in vivo* selection and outline how iterative, feedback-based selection strategies can refine enrichment across multiple rounds. Taken together, our work establishes a high-throughput selection framework integrated into microfluidic devices, enabling applications in directed evolution, biosensor optimisation, circuit engineering, and diagnostics, where selection based on dynamic, multi-trait phenotypes is essential.

Introduction

High-throughput analysis or enrichment of libraries of biodesigns, in the form of “screening”, underpins contemporary biological engineering. Screening capabilities have been dramatically enhanced by single-cell selection technologies, enabling a shift from traditional arrayed screening — where individual variants are isolated in multiwell plates—to pooled screening approaches, in which many millions of variants can be analysed from a single tube. This shift has transformed

multiple areas of biology [1]. For example, the vast increases in throughput have been applied for selection-based directed evolution [2] (contrasting growth-coupled directed evolution [3]), which relies on iterative rounds of selection and mutagenesis to alter the properties of a protein.

One of the most widely used high-throughput selection methods is Fluorescence-Activated Cell Sorting (FACS). In FACS, single cells pass at high speed – up to 100,000 per second [4] – through a laser that excites fluorescent molecules and measures a per-cell intensity. Cells are subsequently sorted into separate collections depending on their fluorescence level. An alternative method, Magnetic-Activated Cell Sorting (MACS), allows interrogation of *external* markers [5]. In MACS, magnetic beads coated with a target-specific binder (e.g. antibody, lectin) are mixed with the sample, where they bind only to cells expressing the target on their surface. Selection is subsequently performed by passing the sample through a magnetised column. Both FACS and MACS are capable of screening individual cells at high throughput, however they are restricted to static phenotypes (as they only measure each cell once), and selecting based on multiple and/or dynamic traits remains a major challenge [6, 7]. Where FACS is often applied for intracellular phenotypes, and MACS for surface-based phenotypes, *secreted* phenotypes can be selected upon using droplet-based microfluidics [8]. In droplet-based microfluidics, cells are encapsulated in small droplets that act as microreactors. These droplets can also be combined with various substrates, making this system a suitable tool for the directed evolution of secreted enzymes [9]. Examples exist of droplet based-microfluidics being used to measure dynamic responses by immobilising drops [10], however, at significantly lower throughput. Long-term observation of cells in droplets presents a challenge due to the finite amount of contained nutrients, as well the complexity of tracking of single cells in three dimensions.

An alternate family of approaches for high-dimensional single-cell measurements, including during continuous growth/culture, utilises “mother machine” microfluidic devices [11] (Fig. S1 [12]). The mother machine design consists of a central media channel lined with up to $\sim 10^6$ trenches [12], each the width of a single cell. Once loaded into the trenches, the “mother cells” at the closed ends remain effectively static over hundreds of generations, allowing long-term observations. Due to physical separation of cells there is no growth-based competition or selection within the device. In this configuration, dynamic phenotypes can be tracked across many generations at acquisition rates limited only by microscopy and camera measurements (e.g., > 100 Hz on standard hardware). Sequential responses to light, chemical, or other environmental stimuli can also be measured and assayed under highly controlled conditions, in ways not possible with other platforms. To process the resulting data, several computational pipelines have been developed for automated segmentation and tracking of mother machine experiments, making high-dimensional datasets readily accessible [13].

Given the rich phenotypic data that can be collected from mother machine studies, physically selecting variants is of significant interest for unlocking downstream applications. It has been shown possible to link phenotypic to genotypic data in the mother machine using dCas9 libraries [14] as well fluorescence *in situ* hybridisation (FISH) [15]. These methods are effective for screening, however they require genetic modifications, extensive sequencing, and in the case of FISH, the cells themselves are destroyed in the process. One approach to extracting cells without the need for genetic modification or destruction is with the use of optical tweezers [16]. This method is able to select a small number of cells from the mother machine, however the process requires a long time period per cell and has only been achieved at low-throughput (e.g., on the order of tens of cells), making it incompatible with high-throughput sorting. It was later shown possible to use strategies such as laser-ablation and optogenetically-induced antibiotic resistance to select cells, paving the way for integration with future high-throughput pipelines [17].

In this work, we introduce Microscopic PhotoSelection (MiPS) as a method for fully automated, high-throughput selection of cells from mother machine devices using targeted UV light, as well as using targeted red light in combination with the photosensitiser methylene blue. These methods both utilise a Digital Micromirror Device (DMD) to project targeted, patterned light onto cells at $< 0.5 \mu\text{m}$ resolution. Connected to high-powered LEDs, we demonstrate it is possible to use the DMD to perform selection on populations in mother machines with $> 10^6$ trenches, with the potential to

expand throughput to the limits of microfluidic device sizes. When compared to FACS—the method currently used for similar challenges—our method unlocks the ability to perform selection based on the rich, multi-factorial data available from mother machine experiments, including dynamic responses [18], tracking of sub-cellular localisation [19], or responses to whole arrays of chemical and/or optogenetic stimuli.

Results

Development of Automated Photoselection Platform

To enable high-throughput selection based on dynamic, multi-trait phenotypes we developed an integrated, fully automated platform that couples high-throughput microscopy with microfluidics, real-time data analysis, and targeted optical actuation (Fig. 1C and Fig. S1C). The platform continuously acquires and processes fluorescence images across multiple fields of view (FOVs), quantifies cell-level traits, and applies selection light patterns without manual intervention (Fig. 1A). Under typical operation (10,000 growth trenches per device; 25 FOVs with 400 trenches per FOV; acquisition every 5 min) the control loop must process each FOV within 12 s.

The platform achieves real-time operation by combining automated microscopy hardware (Fig. 1C) with a custom DMD-based actuation system (Section Optics and DMD Integration). Throughput is increased by using 40× magnification to maximise the number of cells per FOV and by acquiring fluorescence images only (used for both cell segmentation and phenotype measurement) to minimise data acquisition time. To enable this data to be processed automatically the DeLTA pipeline [20] was retrained on 3,000 manually annotated fluorescence images [21] and low-level code optimised, reducing per-FOV image processing time from 11.6 s to 2.22 s. In routine experiments without applying selection light patterns, the full loop (stage motion plus processing) requires less than 5 s per FOV, below the image-acquisition interval. Another key enabling component is the custom built excitation and actuation light-delivery system (Section Optics and DMD Integration), which allows precise targeting of cells and the duration of selective projections to be reduced. It provides high optical power while minimising off-target illumination, and achieves a uniform rectangular excitation profile suitable for both quantitative imaging and selective projections.

Characterisation of Growth Inhibition with Light

To optimise cell selection protocols we began by applying patterned dosing to trenches at different intensities and measuring subsequent reductions in cell viability. In all experiments cell trenches in the mother machine were located and segmented using a modified version of the DeLTA pipeline [20] (Fig. 2A). The coordinates for each identified trench are used to generate a DMD pattern that projects light onto targeted trenches. Figure 2B shows a scenario in which UV was projected onto alternating trenches (8.0 Wcm^{-2} intensity and 300 s duration), subsequently reducing the division rate of targeted cells to on average ~10 % that of untargeted cells.

With the goal of finding an optimal trade off between UV dosage and timing, an array of UV intensities and durations was tested (Supplementary Fig. S2). The highest performing selection of conditions (300 s duration) is shown in Fig. 2C. Cells with a division rate of less than 0.4 per hour were deemed to be killed. After targeting with UV, populations generally exhibited bimodal distributions with respect to division rate (one non-growing population and one with similar-to-initial growth population, and few intermediate rates of growth). This is unsurprising in the light of prior investigations *E. coli*'s response to oxidative stress [22, 23], which is a key mechanism of cell killing by UV light [24]. Expression of stress response genes in bacterial populations is highly hetero-geneous [22], while growth rates stay consistent for a wide range of antioxidant defense enzyme activities before sharply falling for very low levels [23]. As the proportion of targeted cells increases, off-target effects also lead to an increase in the proportion of untargeted cells that are killed; this ratio of on:off target killing is displayed in Fig. 2D. The optimal dose in the tested conditions was 8.0 Wcm^{-2} UV 300 s, which resulted in a 2.78× increase in on:off target ratio with respect to cells killed.

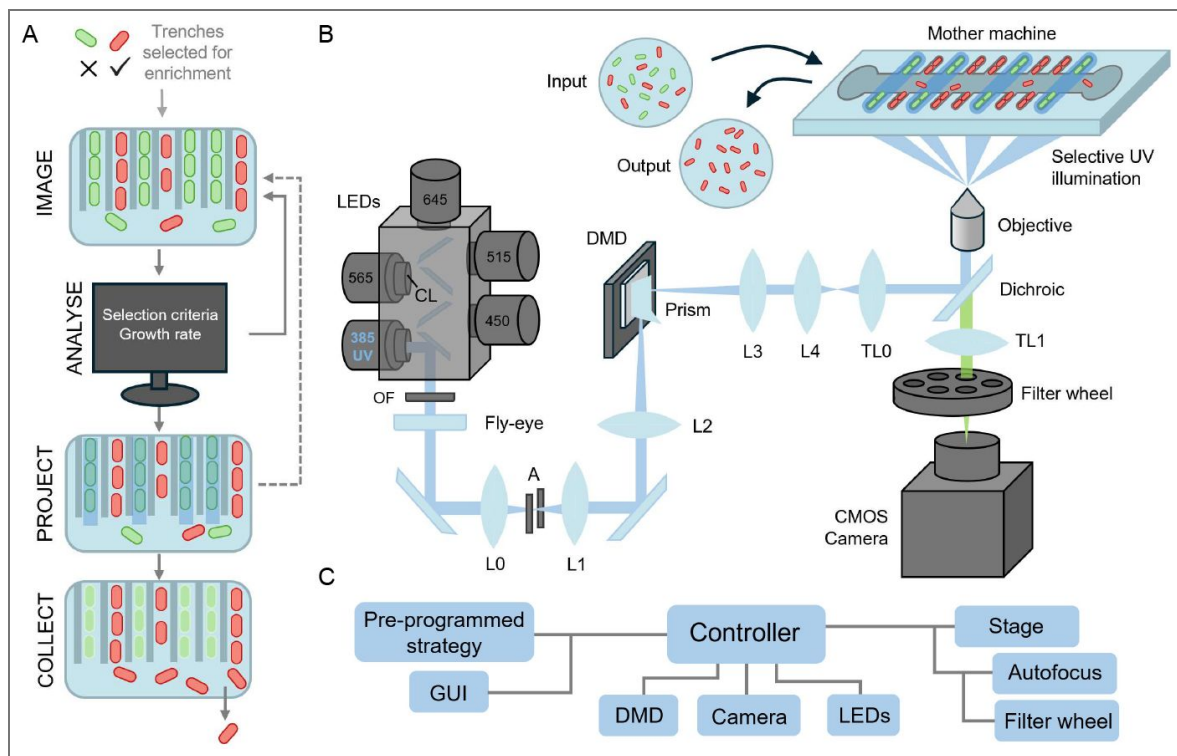


Figure 1. Schematic illustration of the photoselection platform.

A: Automated pipeline for iterative photoselection. After selecting trenches for enrichment, cells are imaged to compute growth rates, which are then used to generate projection patterns that selectively kills cells. This process can be iterated to further refine the selected population. **B:** Hardware outline: Light from a high-power UV LED travels through a fly-eye lens for improved uniformity. A prism positioned directly in front of the digital micromirror device (DMD) ensures that light converges correctly onto the DMD, maximising the area utilised, and ensuring the correct angle of light for reflection into the rest of the optics system. The DMD is an array of micromirrors, which can be individually tilted between two positions (on/off) to dictate the pattern of light projected onto the sample. Patterned UV light travels via three further lenses and is reflected by the dichroic onto the sample, where it damages illuminated cells and prevents further division. When imaging the sample, emission wavelength light travels back through the dichroic, is further refined by the filter wheel, and subsequently detected by the high-speed camera. L = lens, TL = tube lens, CL = collimating lens, OF = Optical Filter, A = aperture, PD = photodiode. **C** Overview of hardware peripherals controlled by the photoselection platform.

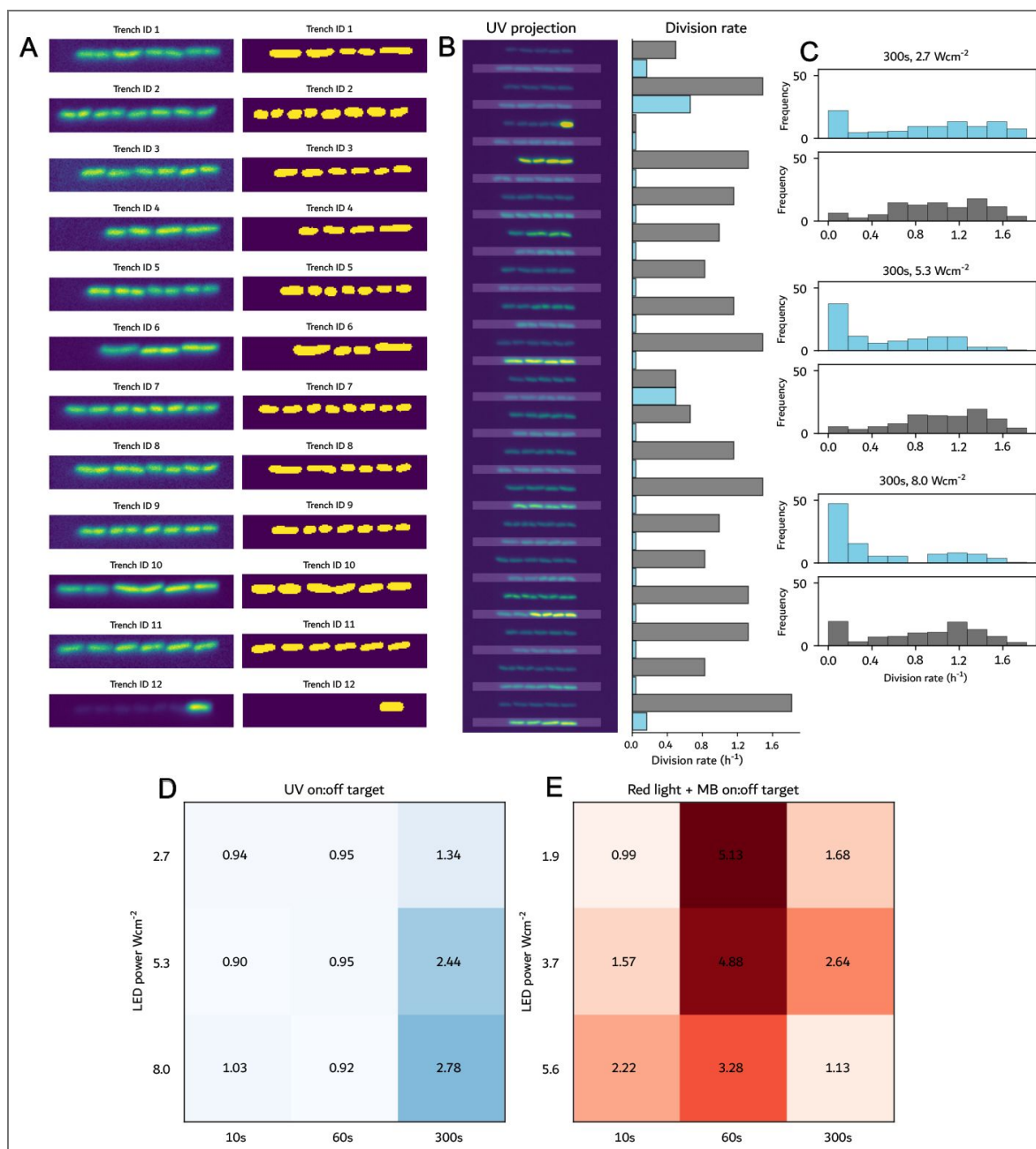


Figure 2. Characterisation of photoselection with UV and red light with the photosensitiser methylene blue (MB).

A: Segmentation of cells in the mother machine by DeLTA, allowing for identification and localisation of cells for UV projection. **B:** Targeting of alternating trenches with UV light projection $\approx 8 \text{ W cm}^{-2}$ (90 % maximum deliverable power) for 300 s (left), and subsequent division rates for each cell (right). Targeted cells division rates highlighted in blue, untargeted in grey. **C:** Impact of increasing intensities of UV light on alternating targeted and untargeted cells. Relative on/off target killing (higher is better) using **D:** UV, and **E:** MB ($2 \mu\text{M}$) with 645 nm red light.

We then explored whether the effectiveness of cell killing could be further improved with the use of a photosensitiser chemical. Methylene blue (MB) has previously been shown to cause production of singlet oxygen species in the presence of red light, which can kill *E. coli* in sufficiently high concentrations [25]. In order to test the efficiency of methylene blue, cells in the mother machine were treated with M9 supplemented with 2 μ M MB for 1 h, followed by projection with red light (645 nm) following the same protocol as used in the UV dosage experiments (Supplementary Fig. S4). The resultant differences in on:off target killing are displayed in Fig. 2E. By utilising the photosensitiser MB, it was possible to achieve up to 5.13 $\times$ on:off target killing, a 1.8 $\times$ improvement over UV, though the optimum in Fig. 2D appearing on the boundary of achievable conditions suggests higher UV dosages may lead to improved performance. This difference is likely due to the fact that MB has a steeper dose response curve, and thus greater differences between on and off-target effects can be achieved at a given pair of on and off target dosages (Supplementary Fig. S3). In addition, the optimal settings at which this is achieved were far lower, requiring only 60 s and 1.9 Wcm² LED intensity, a 5 $\times$ reduction in duration relative to the UV optimum (Fig. 2F). However, it was also observed that addition of MB to the cells causes a growth rate reduction of $\approx$ 40 % even in cells untreated with red light, and therefore this apparent sensitivity could lead to further issues if subsequent rounds of growth and selection are intended.

Quantification of Off-Target Effects

From the experiments in Fig. 2 it is clear that off-target effects cause unwanted death of untargeted cells, particularly in the UV setting. Therefore, several experiments were conducted to gain a greater understanding of these effects. First, to understand whether these effects are primarily local (i.e., limited by contrast and accuracy of the projected DMD image) or global (i.e., device-wide scattering or delivery of off-target light), an experiment was conducted in which a strip of UV was projected on one or multiple neighbouring trenches, and the division rate of the immediate neighbours assessed (Fig. 3A). It was found that there was no significant difference in division rate between a cell one position away from the targeted trenches versus two positions away (or more), suggesting that off-target effects are *not* primarily caused by local effects arising from blurring of DMD light delivery, and instead occur at a larger or global scale. This provides validation for the design of our DMD and optical system, namely that sufficient local (i.e. pixel to pixel) contrast is achieved in image projection to make this factor insignificant compared to other sources of stray light (e.g. reflection and scattering).

A second experiment to quantify the amount of stray light was conducted using selective targeting of fluorescence excitation light (450 nm) to GFP expressing cells, allowing a direct measurement (through fluorescence) of light delivery to each trench (i.e., rather than one mediated by cellular sensitivity to UV measured through growth rate). The assay consists of pairing on-target and off-target measurements of the same trench; in this case an on-target measurement comes from illumination of the target trench (and nothing else), while an off-target measurement comes from illumination of a known area elsewhere in the FOV, and then measuring fluorescence of the target trench arising from excitation by this off-target light (Fig. 3B). The off:on target ratio is then computed by comparing the fluorescence of each trench by off-target light to its on-target fluorescence measurements. A sample of such comparisons is displayed in Fig. 3C. This procedure was conducted across 9 different FOVs, in which an increasing proportion of trenches was illuminated in the off-target tests. 20 individual trenches were measured in each of the FOVs, and the results are shown in Fig. 3D. The ratio of off:on target effects increases approximately linearly with respect to the area of light delivered, and are constant with respect to LED intensity. The exact off:on target ratio expected can be calculated using the proportion of cell trenches targeted, using scale factor 0.26 (the gradient of the linear fit in Fig. 3D). For instance, if 99 % trenches are targeted, then the untargeted 1 % will receive the $99 \times 0.26 \approx 25.7$ % of the on-target dose.

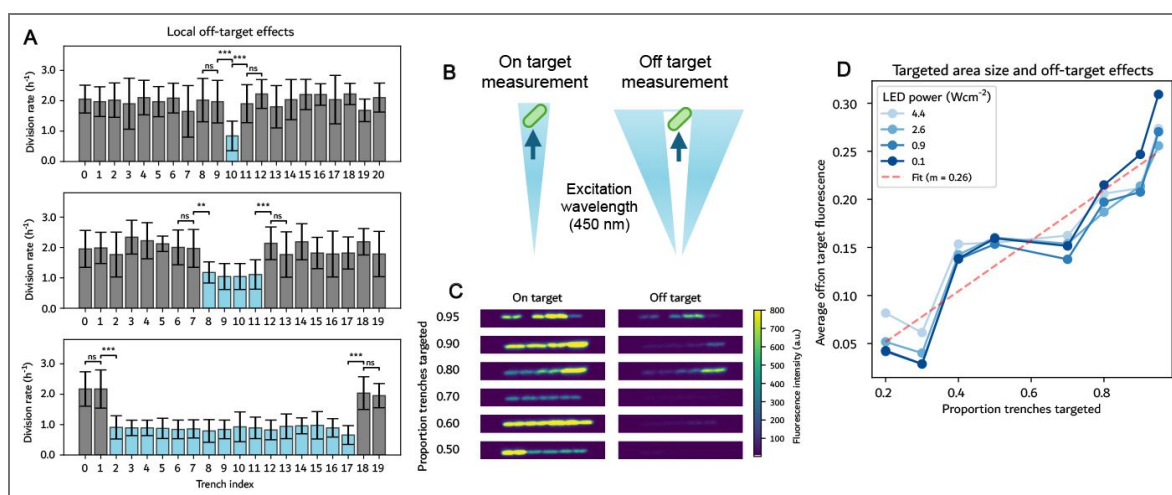


Figure 3. Characterisation of off-target effects.

A: Experiment to explore local off-target effects, in which an increasingly large strip of UV light was projected down the centre of a sample, and the division rates of the immediate neighbours measured. **B:** Schematic of an experiment to explore global off-target effects using the excitation wavelength of GFP. An on-target measurement is from illumination of that cell only. An off-target measurement is illumination of an area away from that cell, but subsequent measurement of any excitation that is a result of off-target light. **C:** Demonstration that as the proportion of trenches illuminated increases, the off-target illumination also increases. **D:** Quantification of the relationship between targeted area size and off-target light. Gradient (m) w.r.t proportion cell trenches targeted = 0.26.

Implementation of Enrichment by Photoselection

In order to test whether the process of selective UV illumination can effectively lead to downstream enrichment of a desired phenotype, an experiment was set up to test whether mCherry cells could be selectively enriched from a mixed mCherry/GFP population. Immediately before loading into the mother machine, cells were combined in an approximate 1:1 ratio. After loading, cells were allowed to grow for several hours in order to achieve homogeneous trenches (i.e. each trench either contains GFP-or mCherry-expressing cells). The mother machine device was then imaged both at 450 nm (for GFP) and 565 nm (for mCherry) excitation, with emission filters complementary to the respective fluorescent proteins. A composite image was used to perform segmentation with DeLTA, and the resulting masks for each trench were compared in both imaging wavelengths to classify trenches as GFP or mCherry (Fig. 4A [↗](#)). The resulting classification was used to generate a projected image specifically targeting GFP (or empty and mixed) trenches (Fig. 4B [↗](#)) for killing. UV was projected in this image at 5.3 Wcm^{-2} intensity for 5 min. The resulting mother cell length traces in Fig. 4C [↗](#) show targeted growth inhibition of GFP cells, indicated by a flat line after the projection at time point 40.

Following validation that growth could be selectively inhibited on the mother machine device, the population flowing out of the device was analysed. In order to minimise contamination from unselected cells, all microfluidic tubing (post-filter) was replaced with fresh, sterile tubing. The population flowing out of the device was collected for a period of 30 min before being centrifuged to concentrate cells and plated on agar. Figure 4D [↗](#) displays the resulting colony counts from 48 h post-experiment, and shows a shift in population composition from 1:1 mCherry:GFP, to 3.8:1 after a single round of targeted selection. In an ideal system *no* targeted cells would survive selection (in which case this ratio would be much larger). However, as illustrated by our characterisation experiments above this outcome is limited by the off-target delivery – if the UV dose is increased further, it will increasingly kill the desired (non targeted) cells and hence not necessarily improve the enrichment ratio.

Simulation of Subsequent Enrichment Rounds

The previous sections illustrate the limitations imposed by off-target effects; below we simulate how these *could* be overcome by implementing multiple rounds of selection to refine the output, i.e. the final ratio of targeted to untargeted cells. Between each round, the division rate of remaining cells is assessed. Within 45 min, 97 % of surviving targeted cells can be correctly identified (Fig. S6 [↗](#)). For subsequent rounds, therefore, only those targeted cells that were not killed are re-targeted, hence reducing off-target impacts on untargeted cells (Fig. 5A [↗](#)). This feedback-based approach succeeds due to fewer cells being targeted in each successive round (e.g. only the survivors), meaning *total* UV dose is reduced each round, preventing off-target effects from killing the desired population.

We modelled this iterative process using a probabilistic UV-killing model fitted directly to the dose-response measurements (Supplementary UV Selection Model) to account for inter-cellular variability in UV sensitivity, which resulted in a sigmoid-shaped survival probability curve as UV dose in-creases (Fig. 5B [↗](#)). At each round of the simulation (Supplementary Implementation of simulation), targeted cells that are still alive are targeted with constant UV doses, which accumulate across rounds because cells are assumed to be re-targeted within 45 min without time for recovery. Off-target UV doses are computed from Fig. 3D [↗](#). Note that the same approach could be used if cells were allowed to recover, which would result in additional selection rounds to meet the same output refinement.

Figure 5C [↗](#) visualises the output of the simulation for a per-round UV dose of 0.6 (corresponding to 3 min at 5.3 Wcm^{-2} UV intensity) and an initial population ratio of 10:1 targeted (green) to untargeted (red) cells. The ratio of alive to dead cells is visualised using the normally-distributed survival potential, where the area over the positive domain corresponds to the fraction of alive cells (Supplementary UV Selection Model). Given the high proportion of targeted cells, the ratio of off-target to on-target light at the beginning is high, at >10 % (calculated from Fig. 3D [↗](#)). With

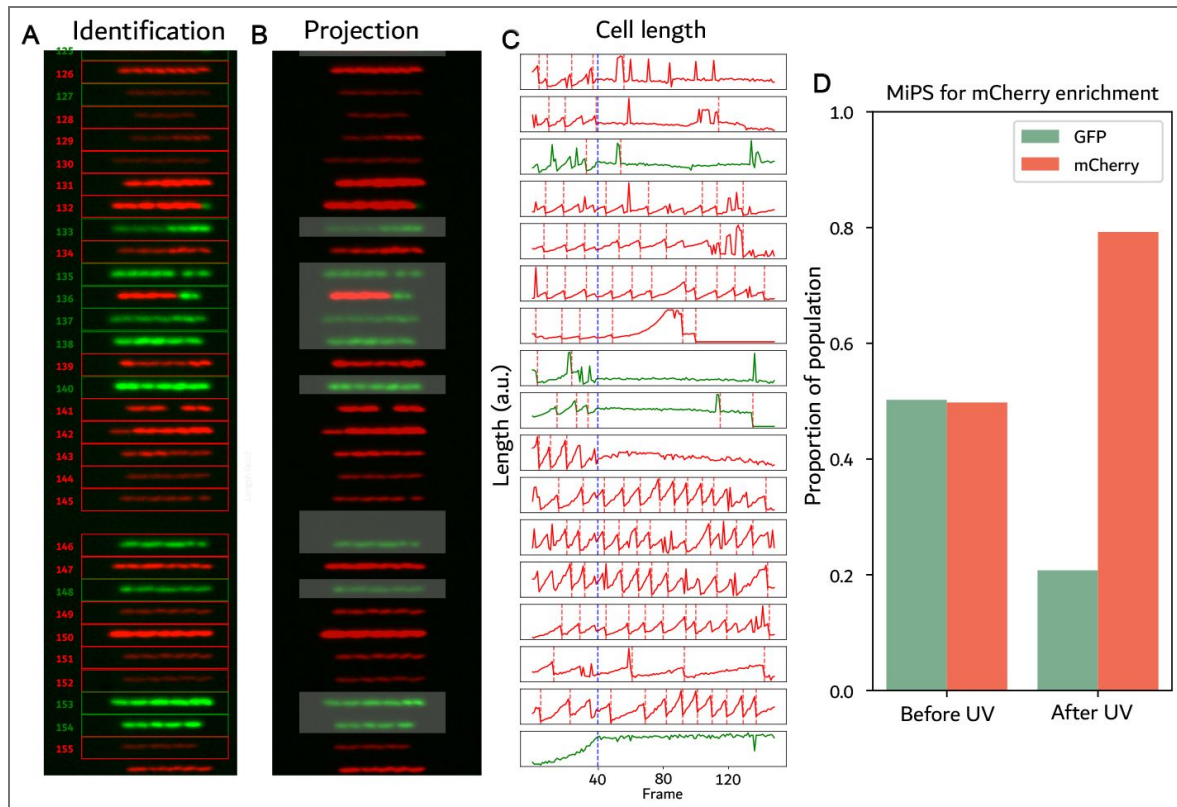


Figure 4. Enrichment of mCherry cells from a mixed mCherry/GFP population by photoselection.

A: Identification of GFP and mCherry cells was carried out by imaging with a 450 nm LED (GFP) and 565 nm LED (mCherry), segmenting the images with DeLTA and comparing the brightness in each channel. **B:** The resulting classifications were converted into a projected image. Unidentified or mixed trenches were projected on by default. **C:** Length of the mother cell in each trench can be used to measure divisions (at which point the cell length sharply decreases, as indicated by dashed red line). When UV projection occurs at time point 40 (dashed blue line), GFP cell length becomes static, indicating no more division, while mCherry cells continue to divide. **D:** Comparison of the population composition within the mother machine device (pre-enrichment), to the population composition flowing out of the device after UV (post-enrichment).

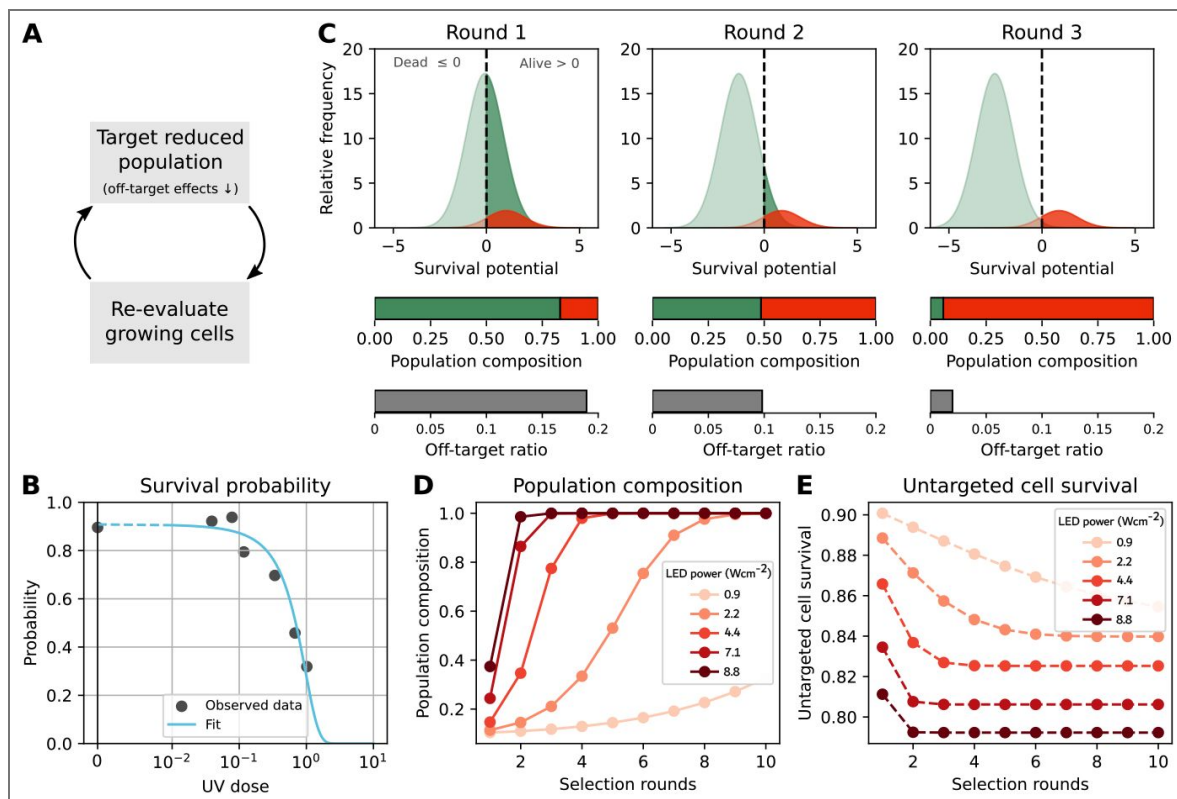


Figure 5. Simulating multiple rounds of targeted selection.

A: Illustrating the two steps of each selection cycle, noting that off-target effects decrease with each round. **B:** Survival probability fit from UV dosage experiments (Supplementary UV Selection Model). One unit of UV dose corresponds to $\approx 8 \text{ W cm}^{-2}$ for 300 s. **C:** Visualising three rounds of simulated selection, including population composition and off-target ratio results from each round. **D:** Population composition and **E:** untargeted cell survival over 10 rounds with different UV intensities.

each round, as the proportion of targeted cells remaining decreases, the amount of off-target light received by untargeted cells becomes smaller, but the targeted population continues to receive *approximately* the same, high dose of UV. This is why the populations appear to move apart with subsequent rounds. After three selection rounds, the fraction of targeted cells remaining in the population is % (a total enrichment of ~ 170 -fold compared to the initial 10:1 ratio), with 0.5 % and 81.9 % of targeted and untargeted cells still being alive. The fraction of remaining untargeted cells could be increased by decreasing the per-round UV dose, at the expense of running additional selection rounds to meet the same output refinement.

This trade-off is further explored in Figs. 5D–E [\[12\]](#) for a starting population of 10:1. With 8.8 Wcm^{-2} per-round UV intensity (the maximum deliverable by our system), one can achieve almost 100 % un-targeted cells after two rounds (Fig. 5D [\[12\]](#)), however over 20 % untargeted cells are lost in the process (Fig. 5E [\[12\]](#)). In contrast, with 2.2 Wcm^{-2} per-round UV intensity, it takes 8 rounds to reach the same population composition, but results in only 14 % loss of untargeted cells. Therefore, one should consider whether their priority is minimising loss of cells desired for selection, or time (45 min between rounds for reliably evaluating growth, up to 2 h per round for UV projection).

Discussion

This work demonstrates Microfluidic PhotoSelection (MiPS) - a method for applying selection to microfluidic chips that leverages real-time data processing and actuation afforded by high capability robotics and real-time image processing algorithms. It was shown possible with a DMD to produce sufficient resolution in a projected image to selectively kill single cells with light. This capability unlocks selection based on dynamic phenotypes in a mother machine context—features fundamentally inaccessible to state-of-the-art workflows such as FACS or MACS.

The current implementation of this platform is applied to chips with approximately 8×10^3 trenches across the whole mother machine device (~ 400 trenches per FOV). This is a limitation of microfluidic device capacity, which can feasibly be increased to the order of 10^6 by using multiple devices in parallel or more densely-packed serpentine designs as opposed to the standard linear channel [\[12\]](#). Serpentine chips have been designed to contain twice as many cells per FOV than the linear designs, and with a lower-magnification objective one could fit up to 1500 cells in a single FOV. While the proof-of-concept selection experiment demonstrated here used 300 s of UV light for selection, it was shown possible to decrease this time to 60 s using higher UV intensities or using the photosensitiser MB alongside targeted red light.

The emergence of two surviving and dying *E. coli* subpopulations in our photoselection step is mirrored by prior studies of oxidative stress [\[22\]](#), caused both by UV and red light in presence of photosensitisers [\[24, 25\]](#). This is explained by populational heterogeneity immediately *prior* to the onset of adverse conditions, with a fraction of cells in favourable environments expressing more stress-response protein at the cost of slower growth [\[22\]](#). In the future, our platform's ability to observe cells over time and measure their growth rates could help identify targeted cells which are stress-resistant. This may allow to overcome the photoselection trade offs, for example having trench-specific non-uniform light intensity delivered by the DMD in proportion to their (estimated) stress-resistance, which would minimise total light delivered (and hence off-target effects) while optimising targeted killing. Such feedback-based dose shaping can be achieved by streaming gray-scale projection patterns rather than binary patterns with our system's existing HDMI implementation. While this proof-of-concept work focused on selection of cells based on a categorical variable (mCherry or GFP expression), future development would apply the technique for selection of cells based on a continuous variable (e.g. fluorescence of a variant library). In this, we believe a further advantage over FACS will be unlocked, which is the time over which cells can be observed. Gene expression fluctuates naturally over time (e.g. as a function of cell cycle phase, intracellular resource fluctuations, stochastic noise in gene expression), and thus single time-point measurements will be inherently noisy. By observing cells over longer durations and averaging the measurements, resulting in a readout closer to the “true” selected phenotype. Beyond simple gene expression measurements, this system also enables exploration of dynamic cellular responses from real-time perturbations. Particularly useful in the context of directed evolution of

biosensors is also the ability to challenge cells with an array of stimuli before performing selection. These stimuli could consist of chemical stimuli flowed in via microfluidics, as well as optogenetic stimuli delivered via the DMD. When applying MiPS to such applications, particularly when the fitness landscapes on which selection is being performed are highly rugged, running the selection process in a regime where some de-selected cells survive each selection round may even *improve* the overall performance of the directed evolution process. This is explored in theoretical studies that highlight benefits of including non-selected cells in subsequent rounds of directed evolution to maintain intra-population variability [26].

Overall our results demonstrate that high-throughput selection from mother machine microfluidic chips can be achieved by integrating a DMD with automated data processing and control algorithms. While mother machines have proved immensely effective tools for understanding dynamic and long-term cell behaviours at the single-cell level, our approach can equivalently be applied to cells on a standard microscope slide when desired phenotypes are compatible with such a configuration. Taken together, these advantages highlight MiPS as an enabler of high-throughput data collection and multi-parametric directed evolution of diverse properties for Engineered Biotechnologies.

Methods

Microscopy Hardware

The custom built microscopy platform (Fig. S1E–F) is based on the Rapid Automated Modular Microscope System (RAMM) from Applied Scientific Instrumentation (ASI). The configuration includes a Nikon PLAN APO λ D 40x/0.95 N.A. objective (part no. MRD70470), a Teledyne Kinetix sCMOS Camera, a five-band fluorescence filter set (405/445/514/561/640nm Quinta Band Set, Chroma part no. 89904), and Nikon Ti-2 200mm tube lens (36mm clear aperture), resulting in a pixel size of $0.1625 \mu\text{m} \times 0.1625 \mu\text{m}$ and a field of view (for the camera's $3200 \times$ sensor of $6.5 \mu\text{m}$ pixels) of $0.52 \text{ mm} \times 0.52 \text{ mm}$. The stage, filter wheel, and hardware autofocus system (ASI CRISP, augmented with periodic software/camera-based autofocus checks) were driven by an ASI TG-1000 Controller. The light source includes a custom-built synchronisation electronics board (based on the Teensy 4.1 ARM Cortex-M7 microcontroller) and a custom-built LED driver system which can provide up to 70 W of power to each of up to eight LEDs channels with hardware-based feedback control of LED current or optical output power. The system was configured with five LEDs with wavelength centres at 385/450/515/565/645nm (Luminus Devices part numbers CBM-50X-UV-Y31-FA380-22, SBR-70-B-R75-KG301, PT-39-G-L51, CBT-90-CG-L11-G100, and PT-39-DR-L51-BD100 respectively). LED power was calibrated by projecting a known number of DMD pixels (and hence area) into the microscope FOV which was captured by a Thorlabs PM100D power meter (with wavelength sensitivity calibration set to the middle of each LED's emission band, or 400nm in the case of the UV LED).

Control Software

The process implemented by the control software is shown in Fig. 1A. Cells selected for killing are identified during initialisation (in the case of Fig. 4) based on fluorescence classification, though the platform *also* allows cells to be identified using any arbitrary or dynamic combination of measured traits. The process then iterates between imaging and analysis to compute growth rates. Based on these growth rates, an algorithm autonomously decides which selected cells to target with a fixed dose of UV. For the *in vivo* experiments of this work (Section Implementation of Enrichment by Photoselection), the projection from Fig. 1B is only executed once, but the control system allows the process to be repeatedly executed in closed loop to improve enrichment performance (Section Simulation of Subsequent Enrichment Rounds).

Programmatically, this is implemented in a callback-based framework, similar to existing frameworks such as CyberSco.Py [27], MicroMator [28], or Cheetah [29], but low-level optimised and parallelised for throughput. The main process manages image acquisition, hardware peripherals, and data processing. A user-defined *strategy* module—running as an independent process—is

provided with processed per-cell measurements and returns high-level instructions (e.g., which cells to target), ensuring that strategy evaluation never interferes with acquisition timing. An optional graphical user interface (GUI)—also implemented as a separate process—allows users to configure strategy parameters.

The main process is implemented in Python on a PC equipped with an NVIDIA RTX 3090 Ti GPU (24 GB VRAM) and 256 GB RAM. It interfaces a modified high-throughput version of the DeLTA pipeline [20, 21], and automates and coordinates the microscopy hardware and software components from Fig. 1C [4]. The camera is controlled using pycro-manager [30]. The stage, filter wheel, and the autofocus system are controlled using a Python serial interface with the ASI TG-1000 Controller (building on the open-source repository [31]). A similar interface is used to connect to the synchronisation electronics board (itself running a custom operating in C++), which controls the LEDs, reads measurements from a photodiode in the light excitation path if used, and connects hardware triggers. The per-FOV processing time of 5 s is met *without* hardware trigger functionalities, e.g., between LEDs and camera, and usage of this could further reduce processing time.

The DMD is operated using the Video Pattern Mode of the DLP LightCrafter software [32] and connected to the control PC via 4K HDMI. On the control PC, this HDMI connection appears as a second monitor onto which the patterns are displayed using a lightweight display window, implemented in C using Simple Direct Media Layer (SDL3) and interfaced via a local websocket with the main Python control software. A photodiode placed in the excitation light path can be used to detect the exact moment at which the DMD updates the pattern, allowing fast synchronisation of imaging and actuation if necessary. Additionally, the control software implements a standard software calibration routine that projects a grid of dots on the DMD and records their location on the camera image. It then computes a homography map (specifically, using `findHomography` from the OpenCV package [33]) that is used to warp patterns projected on the DMD to account for any misalignment or distortion arising in the post-DMD optics.

Optics and DMD Integration

The excitation light delivery system balances the need to deliver large optical power to the sample while minimising off-target and stray light. These design requirements are similar to those of modern DMD-based data projectors, where both large light intensities (a bright image) and contrasts (e.g., brightness ratio of on versus off pixels, often quantified in terms of ANSI contrast) are desirable [34, 35]. Good spatial uniformity of illumination intensity (e.g., across the microscope's field of view) is also desirable to ensure both quantitative imaging (e.g., fluorescence excitation intensity is consistent from image centre to corner) and uniform dosing when light is delivered to kill cells.

The light delivery system of the photoselection platform is illustrated in Fig. 1B [4]. Excitation LEDs are first collimated using aspheric condenser lenses (Thorlabs ACL25416U-A), and then combined with dichroics with cut-off wavelengths at 600 nm, 525 nm, 470 nm, 425 nm respectively. The combined beam passes through the excitation filter of the five-band filter set (Chroma, described above) before reaching a custom made two-sided microlens array / fly-eye (16 mm×22 mm external dimensions, 9.5 mm thick, 0.6 mm×1.0 mm microlens dimensions, ~ 5.9° by 12° degree beam divergence) to create a uniform rectangular illumination profile [36–38] (contrasting the gaussian-like collimated beam intensity) of approximately the same aspect ratio as the DMD. The fly-eye is placed at one focal length (FL) from lens L0 (180 mm FL Thorlabs AC508-180-A) with a broadband dielectric mirror between (Thorlabs BB2-E02, chosen to for good reflectivity at 380 nm). An adjustable rectangular aperture (3D printed) is placed at the focal point between L0 and L1 (200 mm FL Thorlabs ACT508-200-A), allowing the beam shape to be cut to ensure the DMD is not over-filled. This is followed by another lens (L2, 100 mm FL Thorlabs AC508-100-A), with the relative FLs of L1/L2 chosen to create a 4-f relay to de-magnify the fly-eye lens image to fill the DMD's 8.6 mm by 14.7 mm micromirror array. Light arrives onto the DMD through a TIR Prism [39] (adapted from a large industrial data projector) allowing a setup similar to [40]. Use of a prism here allows closer positioning of the optics L2/L3 (as incoming/outgoing beams require 17.5°

angular separation following the DLP670 DMD's mirror tilt angle), which in turn allows shorter focal-length (and smaller diameter) lenses in these positions and hence collection by L3 (75 mm FL Thorlabs AC508-075-A) of a greater amount of the diffracted beam emerging from the DMD (i.e. part of multiple overlapping diffraction orders, see below). In this setup alignment of L2, DMD face, prism, and L3 are critical as the system must collect the reflected light maxima (emerging approximately normal to the DMD on account of the prism orientation) *and* the maxima of the diffraction that arises from the 5.4 μm pitch micromirrors [41] – and in our setup this would ideally be achieved simultaneously for all five wavelength bands. To facilitate this the prism was mounted on a micrometer X-Y translation stage for precise positioning, and the angle and positioning of the incoming beam was adjusted by translating and rotating the *entire* optical setup upstream of the prism (i.e. including L2 through to the LEDs, all of which are mounted on a separate optical breadboard), to align diffraction bands of order 8 at 390 nm, 7 at 450 nm, 6 at 515 nm, 5 at 625 nm, and mid-way between 5th-6th orders at 560 nm (giving this wavelength the worst diffraction/delivery efficiency) at the focal point after L3. The challenge of maximising transmitted power across multiple diffracted wavelengths is addressed in DMD-based projector and lithography systems via a similar approach [41, 42], though in our setup this is made more difficult by the two additional wavelengths. With L3, L4 (AC508-150-A) is chosen to create a 4-f relay system such that, when combined with TL0 (160 mm FL Edmunds Optics # 49-382), the DMD's image is magnified to slightly over-fill the microscope field-of-view visible to the camera's 20.8 mm square sensor. A monitor photodiode for DMD synchronisation (described above) is placed at the focal point between L4 and TL0.

Future development of the platform could improve uniformity further by using the DMD's PWM function (e.g. fast switching of each micromirror) to invert the optical transfer function of the optics between it and the sample e.g. to account for reduced transmission in corners of the objective's field of view. By comparing the results of our measurements of cells in microfluidics against projection of static patterns on static, flat fluorescent targets (where the system typically achieves >100:1 ANSI contrast, and note that ANSI contrast metrics are highly dependent on the optical setup and are hence typically orders of magnitude less than on-off contrast ratios [43]), we believe the measured off target effects in Fig. 3 are largely due to scattering and other optical effects emerging from the *sample* (i.e. rather than the projection system). However, if improved optical contrast is desired in the future this might be achieved by introduction of apertures at various points (e.g., immediately adjacent to L2 and L3) which can cut out “ghost rays” (e.g. second order reflections between the optical elements [44, 45]), which in our setup will be particularly significant in the UVA wavelengths where optic anti-reflective coatings are least effective. However, *in general* there are trade-offs between maximising optical power and contrast for such optical designs [46], and even with further optimisation it may remain challenging to improve ANSI contrast to the levels achieved in commercial data projectors due to the large number of optical elements in microscope objectives which are not necessarily optimised for this function (particularly in UVA wavelengths).

Microfluidic Chip Fabrication

Polydimethylsiloxane (PDMS) mother machines were fabricated by first combining elastomer and curing agent from the SYLGARD 184 Silicone Elastomer Kit (Dow: 4019862) in a 10:1 ratio. The mixture was thoroughly mixed with an electric whisk, and poured onto a silicon wafer with the mother machine design (gift from Stephen Uphoff). The mixture was degassed in a vacuum until all bubbles were removed, and then cured for 2.5 h at 65 °C. The cured PDMS was removed from the wafer, devices cut with a razor blade, and inlet and outlet holes punched with a 0.75 mm hole punch (Darwin Microfluidics: PT-T983-07-25). The final PDMS mother machine was prepared for bonding by removing dust with with scotch tape (3M: 7100235316), washing twice in IPA and drying with air. High-precision coverslips (Thorlabs: CG15KH1) were prepared for bonding by sonicating in acetone for 10 min, washing twice with deionised water, then sonicating in isopopropanol for 10 min. To bond mother machines to coverslips, both were treated in a

Henniker HPT-100 plasma oven at 50% power, 0.3 mbar for 30 s. Bonding was performed by transferring each coverslip treated face up to a hot plate at 80°C, then placing the mother machine face down on top and applying even pressure. Bonded chips were baked at 95°C for one hour.

Media and Strains

All strains used in this work were MG1655, transformed with pVS-01-01-mCherry (a constitutive mCherry expressing plasmid) or pVS-01-03-eGFP (a constitutive eGFP expressing plasmid). All M9 media (Formedium: MMS0102) used in this work was supplemented with 50 µg/ml Carbenicillin (Formedium: CAR0005), 0.4% glucose (Formedium: GLU03), 2 mM $MgSO_4$ (Sigma Aldrich: M7506-500G), 0.1 mM $CaCl_2$ (Sigma Aldrich: C5670-500G), 0.2% casamino acids (Formedium: CAS03), 0.2g/l Pluronic F-127 (Sigma Aldrich: P2443-250G), 50 mg/l EDTA $Na_2 \cdot 2H_2O$ (Sigma Aldrich: E4884-100G), 30.8 µM $F eCl_3$ (Sigma Aldrich: 157740-100G), 6.16 µM $ZnCl_2$ (Sigma Aldrich: 208086-100G), 0.76 µM $CuCl_2 \cdot 2H_2O$ (Sigma Aldrich: 307483-500G), 0.42 µM $CoCl_2$ (Sigma Aldrich: 232696-100G), 1.62 µM H_3BO_3 (Sigma Aldrich: B0394-500G) and 0.081 µM $MnCl_2 \cdot 4H_2O$ (Sigma Aldrich: 221279-500G).

Microfluidic Experiments

Overnight cell cultures were prepared in 5 ml M9 media. The following day, mother machines were injected with 15 µl of 8.5 mg/ml Pluronic F-127 (Sigma Aldrich: P2443-250G), using a gel loading tip (Fisher: 11927734). After approximately 10 min, the overnight cell cultures were injected into the chip in the same manner. In order to load cells into trenches, the chip was spun in a centrifuge at 3000 RPM for 3 min on each side, at an angle of 10°. This was achieved using a 3D-printed chip holder designed to be compatible with a 96-well plate centrifuge rotor (Fig. S1C-D [↗](#)).

The loaded chip was then mounted on a custom-designed microscope stage insert that supports the cover slide around all edges (Fig. S1E [↗](#)). To insert needles for microfluidics, a custom support was designed that provides a backing for the chip to prevent cracking (Fig. S1F [↗](#)). Once connected to the microfluidic pressure flow control system, M9 media with 25% of the standard antibiotic concentration (12.5 µg/ml Carbenicillin) was flowed continuously at a rate of 20 µL min⁻¹. The pressure is controlled by an OB-1 MK3+ Flow Controller (Elve Flow). The OB-1 delivers pressurised air into a 50 ml reservoir containing M9 media. As a result, media is pushed out into the system, which consists of an MFS Microfluidic Thermal Flow Sensor (Elve Flow) which provides flow measurements for feedback control, followed by a 5 cm 100 µm inner diameter resistance element (Darwin Microfluidics: LVF-KTU-05) for more robust flow control. After the resistance element, a 0.2 µm filter is attached to prevent contamination reaching the chip or output. From the filter, the tubing connects directly into the chip via glass capillary tubes (DWK Life Sciences: 764500-0002). Cells were maintained at 37 °C by a custom, blacked-out microscope incubator (Fig. S1G-H [↗](#)), and allowed to grow for several hours before initialising the experiment.

Data availability

All data generated in the study is analysed/presented in the manuscript.

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This preprint was created using the LaPreprint template (<https://github.com/roaldarbol/lapreprint> [↗](#)) by Mikkel Roald-Arbøl.

Additional files

[Supplementary Information](#) [↗](#)

Additional information

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Author ORCID iDs

Kirill Sechkar:  <https://orcid.org/0000-0003-2300-8278>

Jingyu Wang:  <https://orcid.org/0000-0002-2856-7602>

Harrison Steel:  <https://orcid.org/0000-0002-9625-4755>

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors present MiPS, a platform combining DMD-based patterned illumination, automated microscopy, retrained DeLTA segmentation, and mother-machine microfluidics to selectively inhibit or eliminate cells based on dynamic phenotypes. The system enables targeted UV or red-light illumination in real time using segmentation-informed projection masks, allowing selective enrichment directly within mother-machine devices. The manuscript demonstrates proof-of-concept enrichment of mCherry cells from mixed GFP/mCherry populations, characterizes off-target effects, and performs computational simulations of iterative enrichment rounds. Overall, the engineering and systems integration are impressive, and the platform has strong potential for applications in directed evolution, biosensor optimization, and dynamic phenotype-based selection workflows.

Overall, I believe the work is suitable for publication after minor revisions and clarification of several aspects of the manuscript. In particular, the paper would benefit from additional context in the Introduction and Methods sections, clearer positioning relative to existing platforms, improved figure readability/captions, and a more careful revision of the English throughout the manuscript.

Major comments:

- (1) The manuscript should better position MiPS relative to recent microscopy-based and DMD-enabled selection/control systems, particularly Lugagne et al., Nature Communications (2024), DOI: 10.1038/s41467-024-46361-1. That work also combines mother-machine microfluidics, DeLTA-based real-time image analysis, and DMD projection. The key distinction here appears to be physical selection/enrichment through targeted killing rather than optogenetic control, and this difference should be stated more explicitly.
- (2) The manuscript currently compares MiPS mostly to FACS/MACS. However, the more relevant comparison may be recent image-based and microfluidic photoselection systems. A dedicated comparison table discussing throughput, temporal phenotyping, iterative selection, dynamic phenotype tracking, and enrichment capabilities would strengthen the paper.
- (3) The enrichment experiment in Figure 4 represents a relatively simple classification problem (GFP vs mCherry). Since the proposed applications involve subtle continuous phenotypes, it would considerably strengthen the manuscript to include at least one experiment selecting for high vs. low expressors within a single fluorescent reporter population.
- (4) The strongest enrichment result (~170-fold enrichment in Figure 5) is entirely simulation-based. Since the manuscript already states that ~45 min is sufficient between rounds for growth evaluation, a real 2-3-round enrichment experiment seems feasible and would substantially strengthen the platform's practical relevance. This experiment appears realistic within a relatively short time investment.
- (5) The bimodal distributions in Figure 2 suggest that a fraction of cells may be stress-resistant rather than simply surviving randomly. It would be useful to discuss whether repeated rounds could progressively enrich UV-resistant subpopulations.
- (6) The manuscript repeatedly uses the term "killed," although the data shown in Figures 2 and 4 mostly demonstrate strong growth arrest/inhibition. Please clarify how the cutoff of division rate $<0.4 \text{ h}^{-1}$ was selected and whether an independent viability assay was performed.
- (7) The off-target analysis in Figure 3 is one of the strongest parts of the paper and should probably be emphasized more. The conclusion that the dominant effects are global rather than local is interesting, but additional discussion about optical scattering, ROS diffusion, or device-wide coupling effects would strengthen the interpretation.
- (8) UV exposure is inherently mutagenic in *E. coli*, and untargeted cells still receive a substantial fraction of the UV dose at high targeting fractions. Please discuss whether the MB/red-light modality may be preferable in applications where preserving genotype integrity is important.
- (9) The manuscript discusses that methylene blue (MB) improves the on:off target ratio, but MB also appears to reduce baseline growth by ~40% even without red-light exposure. This is potentially important for iterative selection workflows. Please discuss whether this effect is reversible after washout and how rapidly cells recover.
- (10) The manuscript states that the retrained DeLTA model used ~3,000 annotated fluorescence images, but no train/validation/test split or segmentation performance metrics are reported. Since segmentation directly impacts phenotype classification and projection targeting, these details are important for reproducibility.
- (11) The manuscript would benefit from a stronger Methods description regarding DMD calibration, alignment procedures, projection accuracy validation, and computational timing

requirements for the real-time analysis pipeline.

Significance:

General assessment: This is a creative and technically impressive study that combines mother-machine microfluidics, automated microscopy, real-time image analysis, and DMD-based photoselection into a unified platform for dynamic, phenotype-based enrichment. The strongest aspects of the work are the systems integration, the quantitative characterization of off-target effects, and the conceptual demonstration that dynamic microscopy-derived phenotypes can be linked to physical enrichment workflows.

The main limitations are that the biological validation remains largely proof-of-concept and the most compelling enrichment results are currently simulation-based rather than experimentally demonstrated across multiple rounds. In addition, the manuscript would benefit from stronger positioning relative to recent image-based and DMD-enabled microfluidic control systems.

Advance: The study extends the field of single-cell microfluidics and image-based selection by introducing a platform that links longitudinal microscopy measurements directly to physical enrichment decisions within mother-machine devices. To my knowledge, the combination of iterative feedback-driven selection, DMD-based targeted elimination, and dynamic phenotype tracking in this context is novel.

The closest related systems appear to be recent DMD-enabled mother-machine platforms for real-time optogenetic control, particularly those reported by Lugagne et al. (Nature Communications 2024, DOI: 10.1038/s41467-024-46361-1). However, MiPS introduces a distinct conceptual advance by using patterned illumination for selective enrichment/elimination rather than gene-expression modulation alone.

The advance is primarily technical and conceptual, with potential downstream applications in directed evolution, synthetic biology, biosensor engineering, and dynamic phenotype screening workflows that are difficult or impossible to implement using FACS alone.

Audience: The work will likely be of strongest interest to researchers working in synthetic biology, microfluidics, single-cell analysis, systems biology, bioengineering, and automated microscopy. It may also be of broader interest to communities developing dynamic phenotype screening technologies, closed-loop biological control systems, and next-generation directed evolution platforms.

The audience is likely specialized but multidisciplinary, spanning both engineering-oriented and biology-oriented researchers. The methods and conceptual framework may also influence future development of automated selection systems beyond the specific mother-machine context.

Expertise - My expertise includes: Microfluidics, Synthetic biology, Single-cell systems, Automated microscopy, Real-time image analysis, Bioengineering platforms, Dynamic phenotype characterization.

<https://doi.org/10.7554/eLife.112391.1.sa3>

Reviewer #2 (Public review):

Summary:

In this manuscript, the authors reported Microscopic PhotoSelection (MiPS), a closed-loop automated robotic platform designed to link time-resolved imaging with physical sample recovery in mother machine microfluidic devices. By pairing a standard mother machine

layout with a custom DMD optical path, an LED array, and an optimized DeLTA deep-learning model, the system tracks dynamic single-cell phenotypes and isolates specific cells via automated, targeted phototoxicity, i.e. selection by elimination. This is a novel technical development that addresses a clear limitation of snapshot sorting methods like FACS or MACS when screening for time-resolved, lineage-dependent traits. However, several methodological limitations and presentation errors must be addressed before publication.

Major Comments:

(1) Definition of 'Optimal' Dose (Figure 2D): The authors identify $8.0 \text{ W} \cdot \text{cm}^{-2}$ UV light for 300s as the optimal condition. However, this data point lies at the absolute boundary of the tested parameter space. In classical dose-response characterization, an optimum is defined by a local peak or a plateau followed by a decline in performance (typically due to rising off-target toxicity or scatter). Because the performance curve has not rolled over, this represents a boundary condition rather than a demonstrated mathematical optimum. The authors should either extend the parameter sweep to locate the true peak or soften their language to reflect that this is simply the highest performing condition tested.

(2) UV Exposure Time Gap: The exposure time sweep skips directly from 60s to 300s. While the closely spaced early timepoints are appropriate for capturing initial cell-death kinetics, the large gap to 300s leaves a significant engineering blind spot. Figure 3D demonstrates that off-target scattering damage scales linearly with cumulative light energy. If complete target cell arrest can be achieved at an intermediate exposure (e.g., 120s, 180s or 240s), operating the system at 300s unnecessarily subjects neighboring "surviving" cells to secondary global UV stress via device-wide scattering. An intermediate temporal sweep is recommended to optimize the selection window and properly balance target lethality with background library viability.

(3) Baseline Chemical Toxicity of Methylene Blue (MB): The photosensitizer workflow shows a clear improvement in contrast at lower power densities and exposure times. However, lines 151-153 note that the addition of 2 μM MB alone, even without light activation, stunts the baseline bacterial growth rate by $\sim 40\%$. This is a major biological confounder. For applications like directed evolution or dynamic physiological screening, introducing a chemical stressor that nearly halves fitness imposes an unintended selective pressure. This baseline stress may activate pathways that mask or alter the phenotypes of interest. The authors must expand their discussion on how this baseline toxicity impacts multi-round iterative selections, and should ideally evaluate lower concentrations (e.g., 0.5 μM or 1 μM) or alternative photosensitizers to identify a more viable operational window.

(4) Negative Selection Framework and Search Space Scale: The MiPS platform relies entirely on negative selection by destroying unwanted variants. While effective for the demonstrated 1:1 binary proof-of-concept mixture, negative selection scales poorly when screening for rare variants within large libraries. For instance, isolating a single high performer from a library of 105 cells requires the system to successfully target and kill 99,999 individual cells; any statistical leak or failure in killing efficiency directly leads to heavy contamination of the recovered sample. The Discussion section requires a quantitative evaluation of these search space constraints, outlining how they limit the system's utility compared to positive selection mechanisms (such as optical tweezers or droplet sorters) when scaling to rare mutations (<1 in 104).

Significance:

This study presents a significant methodological advance in single-cell analysis and microfluidics by integrating long-term live-cell imaging, automated image analysis, and phenotype-guided cell recovery into a closed-loop platform. Existing approaches such as FACS and MACS are largely limited to endpoint or snapshot measurements, whereas MiPS enables

selection based on dynamic and lineage-dependent cellular behaviors, thereby addressing an important gap in current single-cell screening technologies.

A key strength is the effective integration of mother machine microfluidics, custom optics, and deep-learning-based tracking into an automated and functional system. While the individual components are established, their combination into a phenotype-driven selection platform is innovative and expands the utility of live-cell microscopy from passive observation to active cell selection. The advance is therefore primarily methodological and technological, with potential to enable future conceptual discoveries in cellular heterogeneity and lineage dynamics.

However, limitations remain regarding scalability, robustness, selection accuracy, and generalizability across biological systems. Additional benchmarking and validation would strengthen the work further.

Overall, the study will be of interest to researchers in microfluidics, single-cell biology, microbial systems biology, bioengineering, quantitative imaging, and synthetic biology.

My expertise is in microfluidics, cell sorting and disease mechanobiology.

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Reviewer #3 (Public review):

Summary:

The work describes an optofluidic automation setup to optically inhibit and enrich selected bacterial populations in confined microchannels through negative selection using light stimulation. The work is well described and the manuscript is well constructed.

Major comment:

The authors reported that methylene blue with 2uM incubation has superior performance than UV light. But it's also noted on line 152 there is an inhibition effect from the chemical affecting ~40% of the growth rate.

It will be noteworthy what is the growth curve or at least the MIC of methylene blue used on the MG1655 E. coli by the authors.

Significance:

The optics part of the work is well described, however the materials and methods details of the biological and microfluidic part can be extended.

Overall, the system demonstrated the practical use of combining microfluidics for enrichment of microbial population as a novel alternative method, despite that the efficiency is currently subpar to conventional methods.

But combining further with deep learning phenotype or growth rate monitoring, the technology represents a new path for phenotypic selection which is also novel that conventional methods cannot offer. The work will benefit readers in applied science seeking for new target enrichment based on optofluidics.

<https://doi.org/10.7554/eLife.112391.1.sa1>

Author response:

General Statements

We are grateful for Review Commons' handling of our work. All three reviewers have delivered technical and informed critiques of the work, while also communicating their great interest, belief in the impact, and uniqueness of our work. Below we set out how we will address the reviewer's comments – we believe we can satisfy all points, except for two of requests from one reviewer for further experimental applications of the platform (that go beyond what was claimed in our manuscript), which are discussed in Section 4 below.

Description of the planned revisions

Reviewer 1:

Major Comments

We will add further review of other DMD-based microscopy systems (point 1), and a table comparing to other sorting/selection approaches (point 2). We will further discuss (with added references to literature and past work) biochemical mechanisms of killing/mutagenesis from both Methylene Blue and UV (Points 5, 6, 8, 9) as well as their linking to device-wide phenomena via optical coupling (Point 7). We will add further explanation and code for the DeLTA image segmentation/tracking software and share on Github (Point 10), as well as methodological detail on setup/analysis pipeline (Point 11).

Minor Comments

We will update presentation aspects in Figures (Point 1,9) and captions (Point 7, 8,10,11), and methodological details relating to size of projection patterns and chip features (Point 2, 3). We will re-fit and provide error analysis for off-target effects (Point 4) as well as replicate details (Point 6). Similar to Major point 11, we will add details for analysis of empty/mixed trenches. As above we will publicly share the DMD/microscope software and DeLTA weights on a new Github project (Point 12). We will also edit throughout for grammar/readability.

Reviewer 2:

Major Comments

We will update statements to clarify that 300s is a maximum viable UV dosage based on time available at each imaging location while maintaining instrument throughput (Point 1), and highlight in the methods why this specific time (and those below it) was chosen (Point 2). We will expand the discussion relating to Methylene Blue's biological effects as requested (Point 3) and also raised by Reviewer 1. We will add quantitative estimates of our technique's viability for more unbalanced libraries, similar to what was done in the current final figure as requested (Point 4), and add a table to compare against other methods such as optical tweezers (as also suggested in Reviewer 1 Major pt 2).

Minor Comments

We will update Fig2F reference (Point 1), and typographic mistakes identified (Point 2).

Reviewer 3:

Major Comment

As also raised by Rev 1,2 we will add discussion of MB off-target toxicity, as well as experimental data relating to its impact on growth (eg as suggested through MIC concentration).

Minor Comments

We will add discussion of statistical tests used throughout where missing, here specifically Fig 3A (Point 1), and add error bars for Fig4D where missing (point 2). We will add further methodological details (along with those requested by Rev 2) for the microfluidic device design.

Description of the revisions that have already been incorporated in the transferred manuscript

As described in other sections, I (Harrison Steel) will carry out all edits/additional analyses requested and welcome any further feedback from editors on these.

Description of analyses that authors prefer not to carry out

There are a couple of reviewer suggestions regarding further experimental development that, unfortunately, I cannot carry out. I would stress that these are points regarding possible extensions and applications beyond the claims we make in the manuscript - that is, they are not requests to repeat current experiments or perform additional controls etc to further verify any of claims in the submitted manuscript, but rather suggestions of how we could go beyond our current claims and scope of work. In particular, these are Reviewer 1's major points 3 and 4 which suggest exploring other applications and longer multi-round experiments to strengthen the manuscript.

The reason we cannot carry out this significant set of experimental studies is that the three experimental authors of the paper (James, Sechkar, Towers, who were PhD students during the work) have now graduated and have gone on to jobs in other institutions/companies where they cannot work with the platform in my lab. Further, one of the postdoctoral co-authors (Kempf) has recently achieved independence as a new group leader and so is also not able to continue working in my laboratory, while another (Wang) is now employed in a different research group working in a different area. As such, my (Harrison Steel's) team lacks the logistical ability (e.g. experimental researchers with expertise in this area) to do the extensions of the work proposed. We are currently recruiting a new PhD student to the team to further develop this platform - however the timeline for them to start, be trained, and meaningfully complete these experiments is >1.5 years, making it challenging for this review process.

I hope this challenging logistical consideration makes clear our circumstances and hence planned revisions above.

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