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Review

Windows of operation as qualitative early-stage design tool for microfluidic (single-cell) cultivations

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Microfluidic cultivation systems have transformed the study of cellular physiology by enabling high spatial and temporal analysis under precisely controlled environmental conditions. However, the successful application of these systems is hindered by technical challenges, including the lack of systematic characterization of key operational parameters and their interdependencies, which limits experimental reproducibility within a given setup and hampers the rational design of new systems. Here, we propose adapting 'Windows of Operation' — a framework originating from bioprocess engineering to visualize how different parameters define design limits — as a qualitative operational design tool for microfluidic cultivation, here further denoted as microfluidic window of operation (MWO). Through selected case studies, we demonstrate how defining MWOs can guide the identification and optimization of key experimental parameters. This provides a foundation for robust experimental design that links device function with operating parameters, thereby advancing feasibility, robustness, and comparability, while minimizing experimental bias.

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Microfluidic (single-cell) cultivation

Driven by an ever-growing demand for high-resolution, quantitative insights into cellular physiologies, microfluidic cultivation systems have become indispensable across the life sciences [1,2]. Microfluidic systems exploit the distinct physics of microscale flow, characterized by laminar regimes and high surface-to-volume ratios. This enables precise generation and manipulation of physicochemical microenvironments at femto- to microliter scales [3,4]. Furthermore, the inherently small scale of microfluidic cultivation systems confers additional advantages, including portability and miniaturization, parallelization, high-throughput experimentation, and reduced reagent consumption [5,6]. Microfluidic cultivations can be categorized into several concepts, including well-based systems [7], droplet-based systems [8], and single-cell cultivation systems [9], each with various subcategories. These approaches can be implemented in different cultivation modes, such as continuous perfusion or quasi-batch cultivation [10]. Combined with integrated sensors, automation, and time-lapse microscopy, microfluidic cultivation systems enable highly spatiotemporally resolved studies of living prokaryotic and eukaryotic cells across multiple levels of biological organization, ranging from complex organ models [11] to individual cells [9,12] while offering precise control over environmental conditions.

Microfluidic cultivation systems are increasingly used in biology to study the dynamics in various cellular physiologies. For example, microfluidic single-cell cultivation (MSCC) systems enable continuous, long-term cultivation and analysis of individual cells with precise environmental control [13,14]. These systems have opened new opportunities in systems biology, synthetic biology, and bioprocess development, offering insights into cellular heterogeneity [15], gene expression dynamics [16], phenotypic diversification [17], stress responses [18], and evolutionary mechanisms such as persistence [19], bet-hedging, and division of labor [20].

Given the diversity of available devices and applications for MSCC, designing, selecting, and operating a suitable system remains a considerable challenge, particularly for new users. Microfluidic devices vary in cell-retention strategies, retention efficiency, and suitability for specific readouts [21]. The influence of the cell trapping strategy and thus the experimental setup itself on cellular physiology must be carefully considered [22]. Balancing the competing requirements of reliable cell retention, controlled nutrient delivery, and unobstructed imaging is inherently difficult, and the lack of standardized design principles limits reproducibility and comparability.

Especially for perfusion-based microfluidic cultivation systems, establishing and maintaining optimal operating conditions remains a major challenge. Despite the aforementioned advantages, the high turnover — or short residence time — prevents the cells from conditioning their surroundings. Consequently, the experimental outcome is highly dependent on the chosen cultivation conditions, which need to be carefully chosen for each organism and microfluidic system, as the interplay between geometry, flow dynamics, and biological constraints is highly context dependent. Even minor deviations can induce disproportionately large effects on cellular physiology.

To address these challenges, we propose applying the windows of operation concept to microfluidic cultivations. This concept was originally developed in bioprocess engineering [23] and later adapted and expanded for sustainable bioprocess operation [24]. Windows of operation define bounded parameter spaces constrained by physical, chemical, biological, and engineering limitations. Applied to microfluidics, this framework enables systematic identification of operational regimes that are both technically robust and biologically relevant, hereafter referred to as microfluidic windows of operation (MWOs). Critically, windows of operation can guide experimental parameter selection during early-stage design, even when only limited empirical data are available, and can adapt dynamically as experimental setups evolve. Some boundaries are sharply defined by physical thresholds, whereas others are gradual, with performance declining progressively beyond the optimal range.

Toward defined microfluidic experimentation

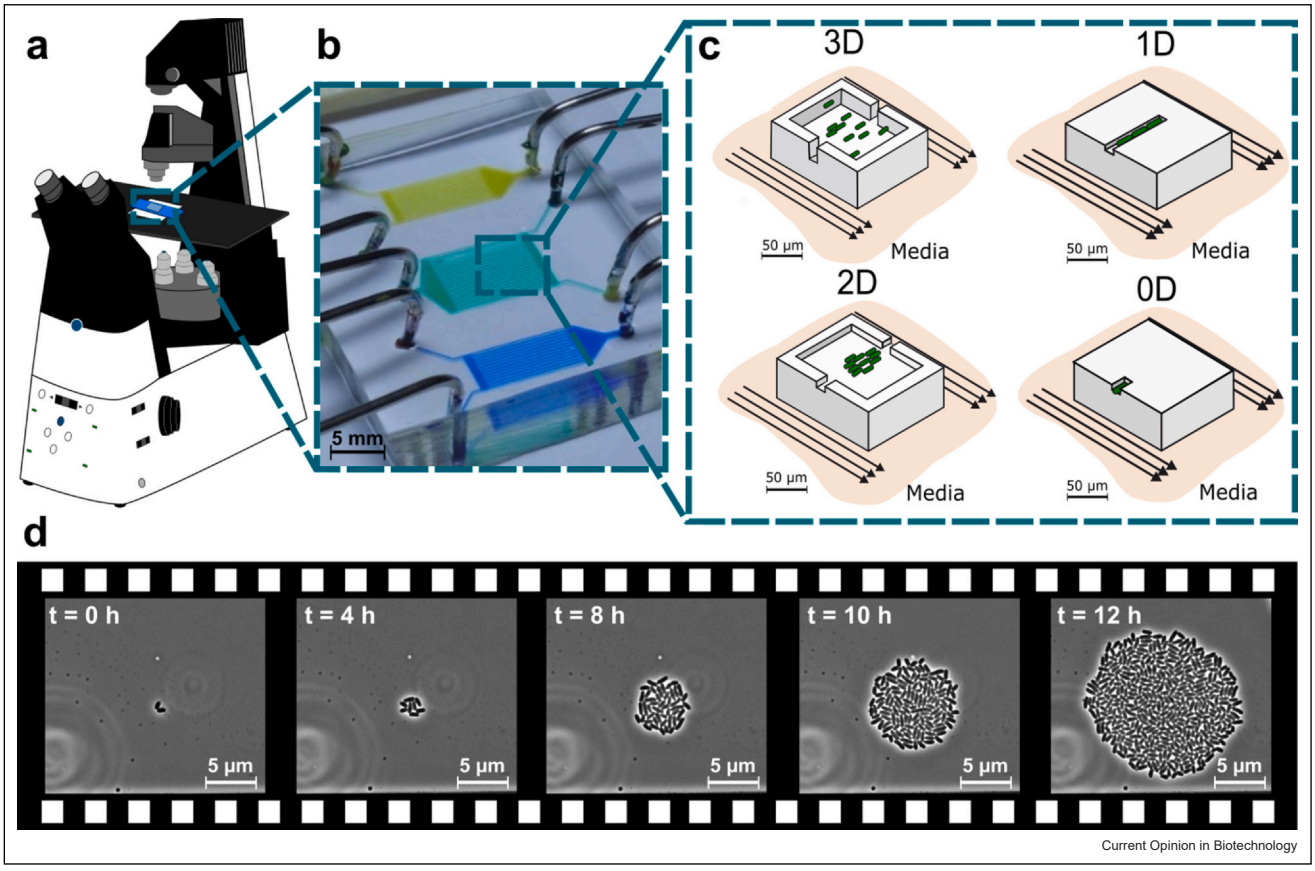
Experimental design and operation in MSCC requires balancing operational parameters, data acquisition, biological performance, and experiment goals and constraints. Once a microfluidic system is chosen or designed (selection or design process is beyond the scope of this review), experimenters need to find a first set of operational parameters. Traditionally, operational parameters have been determined through trial and error or passed down heuristically, adjusted over successive generations primarily to ensure functionality, with systematic studies rarely performed. When complete datasets or detailed system knowledge are lacking, qualitatively assessing the broad operational range provides a practical starting point. While such qualitative MWOs do not eliminate the need for empirical optimization, they transform trial and error into a more targeted, knowledge-driven process, thereby reducing experimental effort and improving feasibility and robustness. Since this procedure strongly depends on the overall class of microfluidic cultivation (e.g. droplet microfluidics, well-based cultivation systems, single-cell cultivation), we here describe the concept of MWOs on the example of perfusion-based MSCC systems (Figure 1) [25]. Their functionality relies on live-cell imaging for data acquisition (Figure 1a). These microfluidic chip systems contain main nutrient channels (Figure 1b) that provide a continuous influx of fresh medium and steady perfusion throughout the device. The main channels are typically connected to hundreds of fl-nl cultivation chambers (3D, 2D) [26], channels (1D) [27], or traps (0D) [28] for cell retention while allowing efficient nutrient supply via diffusion (Figure 1c). Optimal nutrient supply is an interplay between flow rate (FR), nutrient concentration, and chamber size and geometry. Together, these elements ensure that cell physiology can be monitored over time under precisely defined conditions (Figure 1d). For further information regarding design and application, see Grünberger et al. [9].

Identifying microfluidic window of operation key parameters

Defining a window of operation for perfusion-based MSCC begins with two steps: (i) identifying key operational parameters, and (ii) determining the constraints arising from system characteristics ('system constraints') and from the biological system under investigation and analytical requirements ('experimental constraints'). Key operational parameters can be derived by analyzing which variables predominantly influence mass transfer, analytical requirements, and performance within the microfluidic system (e.g. flow rate, acquisition rate, nutrient concentration).

Whereas agitation and aeration are the governing parameters to adjust in stirred-tank bioreactors [23], perfusion-based MSCC with live-cell imaging is dominated in most cases by a different set of key operational parameters. One key parameter is the FR, which determines mass and heat transfer, including nutrients and dissolved gases, and controls pH and other medium-associated parameters. Oxygen can often become a rate-limiting factor in many microfluidic systems, except in devices based on polydimethylsiloxane, where the material's high gas permeability typically ensures sufficient oxygen transfer as given for MSCC [29]. MSCC relies primarily on live-cell imaging as an analytical tool rather than offline analytics [30]. Therefore, imaging settings critically affect the overall experimental setup. For instance, the image acquisition rate, which correlates with the number of recorded positions, represents a critical parameter influencing the interpretability of experimental data (Figure 2a). As the number of positions increases, the acquisition rate per position decreases, since the microscopic system cannot capture all chambers at high temporal resolution. Consequently, when the image acquisition rate is too low, dynamic cellular physiologies such as rapid movements or cell division may

Figure 1



Overview of MSCC platform. **(a)** Live-cell imaging platform. **(b)** Microfluidic device. **(c)** Magnification of different cultivation concepts, namely of 0D, 1D, 2D, and 3D. **(d)** Magnification of cultivation chamber with representative image sequence showing growth of cells in a microcolony.

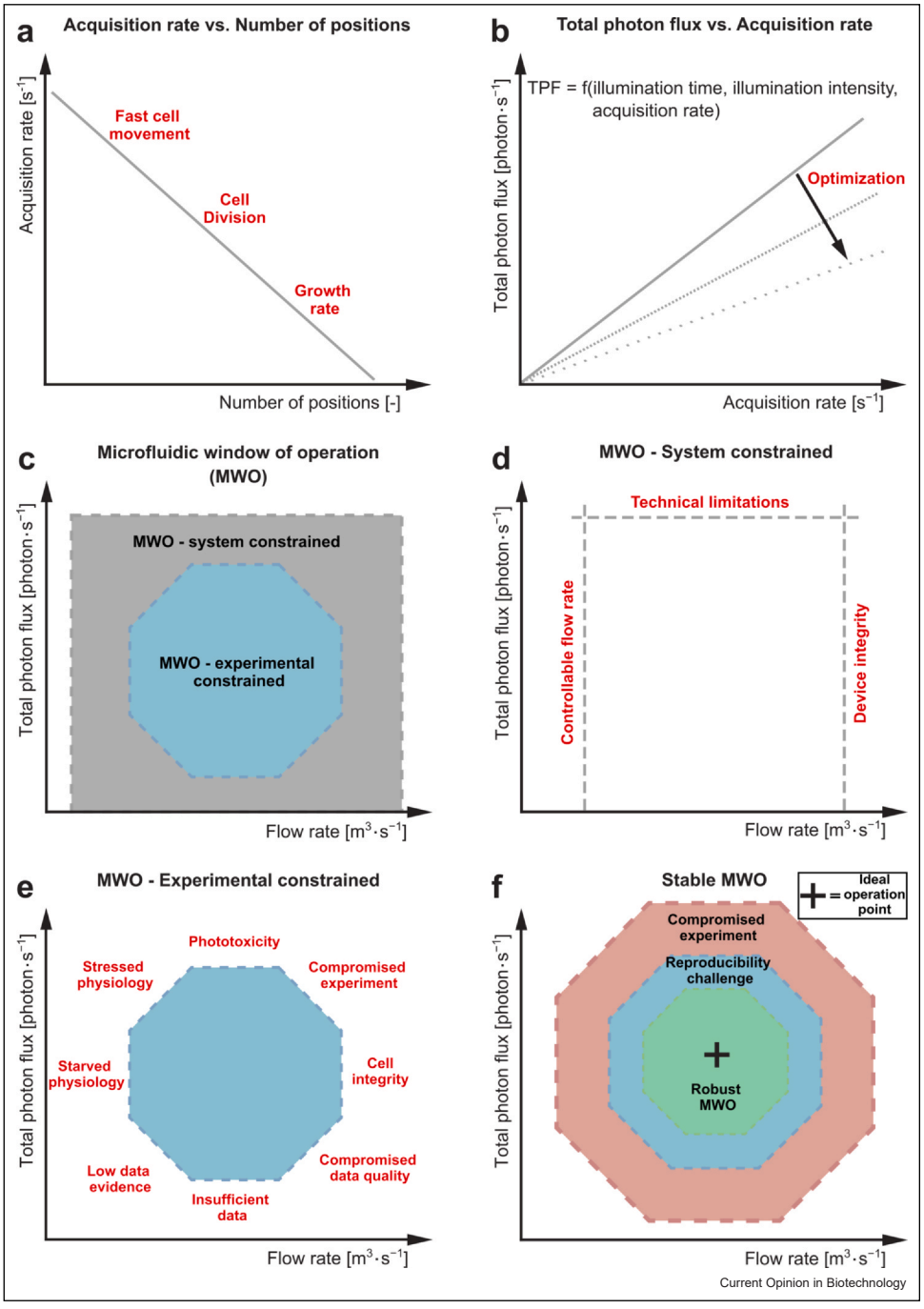
not be adequately resolved. Together with illumination time and intensity, the acquisition rate determines the total number of photons incident on a cell [31]. These factors can be integrated into the total photon flux (TPF), which serves as a proxy and defines a second key operational parameter in perfusion-based MSCC cultivation (Figure 2b). FR and TPF jointly define the physical and analytical framework of MSCC experiments: FR governs nutrient and gas exchange and shapes the mechanical environment of the cells, whereas TPF reflects the analytical resolution. Together, they define the principal axes of the MWO, delineating the range of feasible and reproducible experimental conditions (Figure 2c).

While MWOs are often illustrated in two dimensions for simplicity, real experimental systems are inherently multidimensional. In practice, additional factors such as temperature, dissolved oxygen, shear stress, or wavelength of imaging can also play critical roles depending on the cell type, device design, or experimental objective. Consequently, FR and TPF should be regarded as central but not exclusive parameters within a broader, multidimensional operational landscape.

Mapping and refining the microfluidic window of operation

FR and TPF define an operational window constrained by system and experimental limitations (Figure 2c–e). Stable perfusion ensures adequate mass exchange but is bounded by an upper limit, where device integrity may be compromised (e.g. chamber deformation or leakage), and a lower limit imposed by the periphery, here by the minimal controllable FR of the pumping system. The upper limit of TPF is defined by technical feasibility, particularly acquisition speed, readout time, and data-handling capacity of the microscope system. In contrast, TPF has no lower technical limit, as the photon flux can theoretically be reduced to zero (Figure 2d). Considering the biological system under study, the MWO is further refined (Figure 2e). Concerning FR, the upper limit is defined by cell integrity under mechanical stress (pressure and shear) ('cell integrity') [32], while the lower limit depends on the cells' capacity to tolerate nutrient limitation ('starved physiology')

Figure 2



Concept of the MWO. **(a)** Interconnection of acquisition rate [s^{-1}] and number of chambers [-] recorded. **(b)** Dependency of the TPF [photons·s^{-1}] and the acquisition rate [s^{-1}]. **(c)** MWO, illustrating system and experimental constraints. The operational space is defined by the relationship between two key experimental parameters. **(d)** Operational space restricted by system limitations for FR [m^3·s^{-1}] and TPF [photons·s^{-1}]. **(e)** Operational space restricted by experimental constrained limitations for FR and TPF and selected experimental key parameters. **(f)** Operational space for reproducible MSCC experiment. Within this window, microfluidic cultivations can be conducted under stable and analytically accessible conditions.

[33]. Regarding TPF, the upper limit is imposed by the cells' tolerance to phototoxicity ('phototoxicity') [34], and the lower limit is dictated by the biological question and minimum imaging frequency ('insufficient data') [35]. Experimental conditions at high FR and TPF may compromise reproducibility and interpretability of results due to the cumulative effects of mechanical stress and phototoxicity ('compromised experiment'). High FR combined with low TPF, here caused by low imaging frequency, can lower data quality, for instance, by causing cell loss or displacement that impairs cell tracking ('compromised data quality') [33]. Conversely, low FR and low TPF can reduce data yield and informational content ('low data evidence'). Low FR may promote clogging in main channels, potentially caused by cell accumulation or evaporation-triggered crystallization of chemical compounds in the medium. This may lead to unintended interactions between growing colonies, both in channels and chambers, resulting in obscured cell physiology [36]. Finally, low FR combined with high TPF may compromise cell physiology due to increased phototoxic stress and insufficient nutrient supply for repair processes ('stressed physiology') [37].

Conditions close to the operational boundaries may reduce reproducibility, as cells become increasingly exposed to environmental extremes that trigger metabolic shifts and adaptive responses. Thus, reproducible experiments require defining a robust operation point within the MWO (Figure 2f), which depends on the biological demands of the cells, the microfluidic system, and its periphery (see 'Establishing stable operation point').

Case 1: additional biological demands

The aforementioned flexibility becomes especially beneficial when additional biological demands confine the feasible operating range further. For example, in fluorescence-based monitoring of intracellular metrics such as product formation [38], stress responses [39], or intracellular metabolite content [40], imaging strategies must balance signal-to-noise ratio with the minimization of photobleaching and phototoxicity [41] (Figure 3a,b). In the case of phototoxicity-sensitive cells (Figure 3a), imaging protocols must therefore be optimized regarding frequency, exposure time, and intensity to ensure sufficient analytical resolution while maintaining natural cellular physiology over extended periods (operating point needs to shift from unfavorable conditions (red shaded area) to favorable conditions in the MWO). In the case of cells with a weak sensor signal at low photon fluxes, imaging protocols must be improved in the other direction (Figure 3b). Under these conditions, a narrow window of operation emerges.

Case 2: additional technical demands

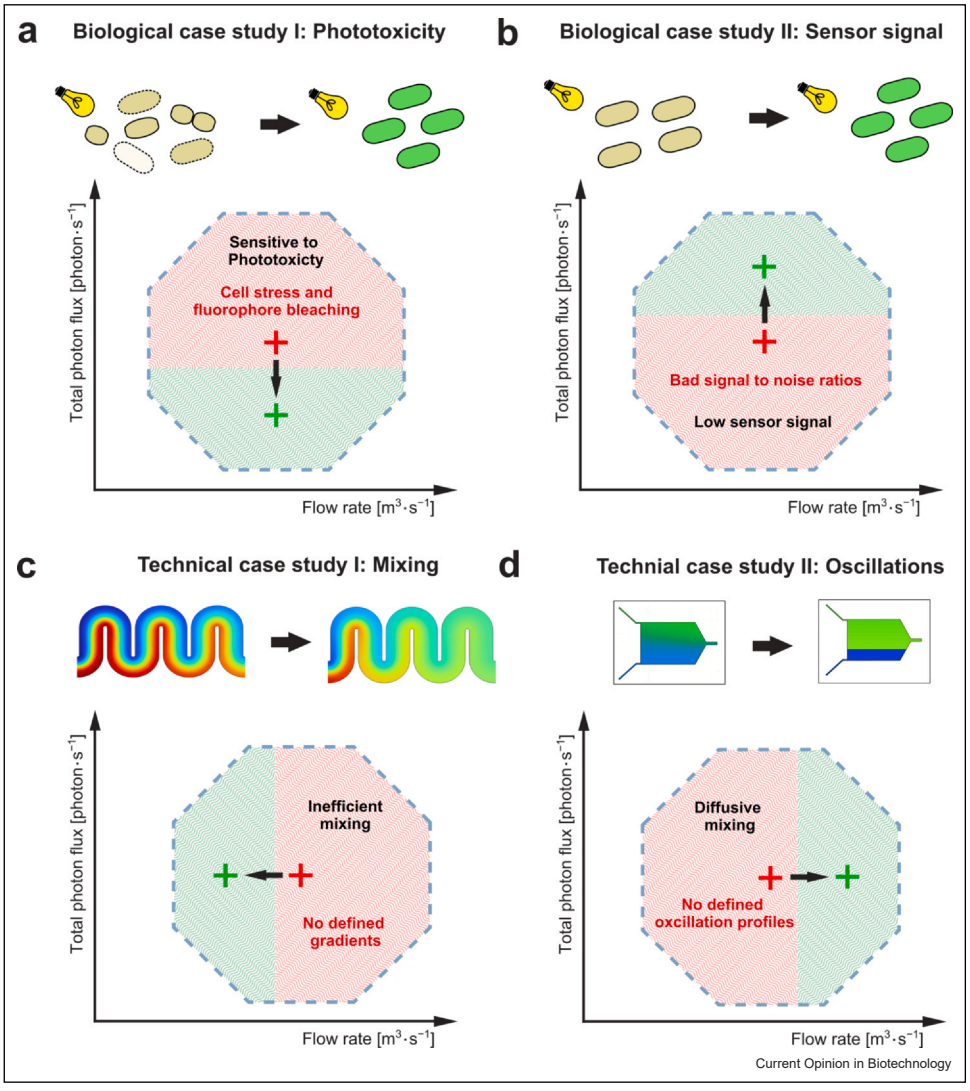
Multiplexed flow profiles [42] and environmental perturbations [43] in microfluidic systems require deliberate modulation of flow conditions, which are often connected to the overall FR, inherently narrowing the window of operation (Figure 3c,d). Such perturbations can be generated through multiple, and sometimes opposing, strategies. An example of this is microfluidic gradient generator systems (Figure 3c). The fundamental functionality of these systems relies on two fluids flowing in parallel under laminar conditions, which intermix by diffusion across their interface, thereby generating distinct concentration profiles [44]. In most cases, gradient generators consist of multiple serpentine channels, which utilize secondary flow effects to enhance mixing. Here, FR constitutes a critical parameter, as excessively high velocities reduce residence time and hinder interfacial diffusion, thereby preventing sufficient mixing of adjacent fluids and compromising the formation of reliable and well-defined gradients. Consequently, the MWO is narrowed, which precludes the operation at high FR (Figure 3c, red shaded area). Another strategy to establish defined environmental perturbations is the use of dynamic MSCC devices, as demonstrated by Täuber et al. [45]. In this system, two distinct fluids are connected to the chip inlets, and controlled pressure differences allow rapid switching between the media with temporal resolutions on the order of seconds to minutes. A key feature of this technique is the laminar flow regime, which prevents convective mixing at the interface and thus maintains sharply separated conditions across the chip system. At insufficient FRs, however, diffusion causes vertical mixing of the media streams, so that distinct conditions can no longer be maintained in downstream cultivation chambers. Therefore, a minimum FR is required to ensure sharp environmental boundaries while avoiding excessive internal pressure and mechanical stress on both the device and the cells. This consideration restricts the operational window of dynamic MSCC systems, as rapid and precise medium switching cannot be achieved at very low FRs. Instead, the effective operating point shifts toward higher flow regimes, where the desired functionality of fast and well-defined environmental perturbations can be reliably maintained (Figure 3d, green shaded area).

These examples show that each microfluidic design, microfluidic cultivation concept, and biological model system has a strong influence on the MWO. These examples presented here are illustrative but not exhaustive. For each combination of microfluidic system and biological model system, functional MWOs need to be established independently. Consequently, defining MWOs for different microfluidic and biological systems represents only the first step; establishing stable and reproducible operation points within these windows is essential to ensure meaningful experimental outcomes.

Establishing stable operation points

Once the MWO has been established, the next step is to identify a stable operation point within this range. Rather than redefining the MWO itself, this step focuses on selecting a specific combination of parameters at

Figure 3

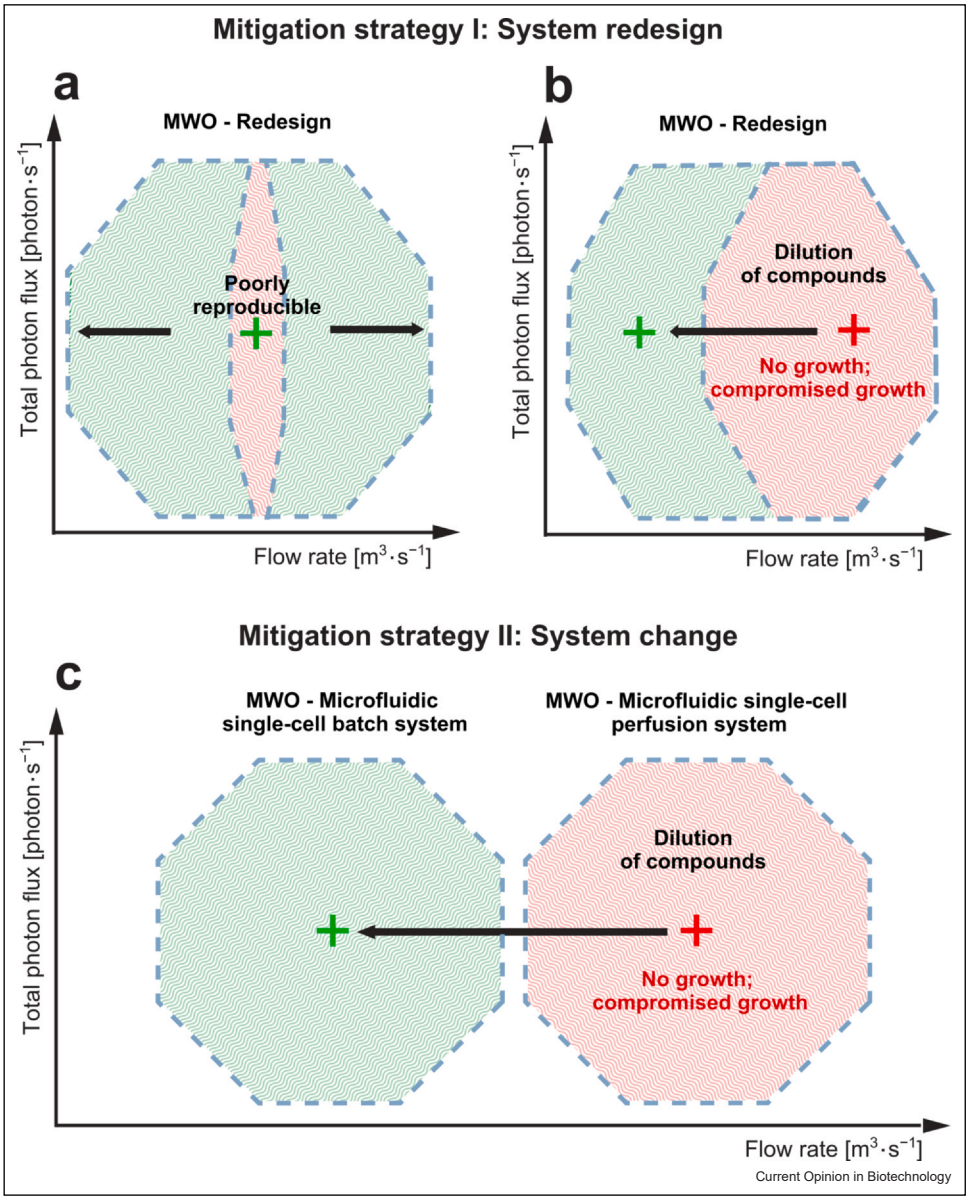


Biological and technical constraints in microfluidic cultivation and imaging. **(a)** Biological case study I: Phototoxicity. Excessive light exposure during fluorescence imaging can induce phototoxic effects, including cell stress and fluorophore bleaching, limiting experimental conditions despite the need for high photon flux. **(b)** Biological case study II: Sensor signal. Low light exposure reduces sensor signal, leading to poor signal-to-noise ratios and weak sensor signals. **(c)** Technical case study I: Mixing. Inefficient mixing prevents the establishment of well-defined gradients, restricting precise spatiotemporal environmental control. **(d)** Technical case study II: Oscillations. Diffusive mixing in microfluidic devices impairs defined flow profiles, especially under fast environmental changes, resulting in poorly controlled oscillation dynamics. The shaded red areas indicate problematic regimes (biological or technical limitations), while the blue dashed boundaries highlight the operational parameter space defined by FR and photon flux. Red and green crosses denote the original and new ideal operation point, favorable and unfavorable conditions, respectively.

which the system remains robust and reproducible, even under minor perturbations. The applicability of the MWO framework is ultimately constrained by the compatibility of the system with the intended biological objectives.

If the MWO is narrow, the system becomes highly sensitive to external perturbations. Even minor perturbations can shift the operating point outside this window, potentially compromising the experimental outcome. To mitigate this, a redesign of the microfluidic system that broadens the operational window could enhance robustness and reliability under varying operational conditions (Figure 4a). For example, in dynamic MSCC systems, the position of the laminar flow boundary is precisely defined by a specific pressure ratio. However, pressure fluctuations can shift this boundary, potentially affecting local environmental conditions. Increasing the distance between cultivation arrays could broaden the operational window by reducing the system's susceptibility to

Figure 4



Mitigation strategies where no robust or feasible operating point can be identified within the MWO of the applied system. **(a)** If the MWO is too narrow, it is advisable to adapt the microfluidic system to achieve a lateral expansion of the operating window. **(b)** Redesign of the system could be necessary when low FR could only be achieved by the system geometry, instead of turning down the pumping capacity. **(c)** If no operating point can be identified within the MWO of the applied system, a switch to a different platform with an alternative operating principle may be required (e.g. from a perfusion-based to a batch system). The shaded red areas indicate problematic regimes (biological or technical limitations), while the blue dashed boundaries highlight the operational parameter space defined by FR and photon flux. Red and green crosses denote the original and new ideal operation point, favorable and unfavorable conditions, respectively.

pressure variations [46]. Another example of compromised cellular physiology is the disruption of quorum-sensing or other signaling processes caused by excessive mass transfer at high FRs. Simply lowering the pump speed may be insufficient to restore physiological conditions, due to diffusion-convection inbalance. Instead, appropriate design adaptations such as modifying channel geometry or adding flow resistors are required to promote signal retention (Figure 4b) [47].

When the operating mode and its associated MWO are misaligned with the experimental requirements, system performance becomes unstable or inconsistent. Such misalignment is often indicated by systematic deviations, high

variability between replicates, or reduced reproducibility under identical conditions. In these cases, switching to a different system might be necessary, such as moving from a perfusion-based MSCC to a batch cultivation approach [48] or adopting an entirely different microfluidic platform (Figure 4c).

Redesign and system changes illustrate how operational parameters and device design can be tuned in tandem to expand the accessible parameter space for stable MWOs. In practice, such adaptations typically allow only a lateral shift of the operating window along the *x*-axis (FR), whereas changes in the vertical dimension (TPF) would require the implementation of an advanced imaging setup. If both strategies fail, alternative approaches can be tested, including modification of the medium composition [49–51]. In the case of cells that rely on quorum sensing, supporting or mimicking essential signaling cues, such as quorum-sensing molecules or metabolic cofactors that stabilize cellular physiology, could be one option. Ultimately, defining a stable operation point within the MWO ensures that technical feasibility, analytical resolution, and biological performance remain balanced, providing a robust foundation for reproducible microfluidic experimentation.

Challenges and emerging fields

Identifying critical parameters that determine the performance of microfluidic systems and cultivation processes remains a major challenge. Qualitative mapping of MWOs can provide an initial conceptual framework to visualize process relationships and potential sources of variation. However, transitioning from such qualitative representations to quantitative MWOs is labor-intensive and requires extensive experimentation, precise parameter quantification, and iterative refinement under controlled conditions. These efforts are often resource-demanding, limiting their applicability in early-stage investigations or laboratories with restricted technical infrastructure. In this context, design-of-experiment (DoE) strategies, well established in bioprocess engineering but rarely applied in microfluidic cultivation, offer a systematic and efficient approach to identify critical parameter ranges and reduce experimental workload [31,52]. Combining qualitative MWOs as a knowledge-based foundation with quantitative DoE-driven optimization could thus provide a powerful pathway toward rational and resource-efficient microfluidic process development. The first integrated optimization, including operation and design parameters for microfluidic applications in biomedicine, has been reported by Atsani et al. [53].

While the MWOs presented in this study are depicted in two dimensions, focusing on two dominant rate-limiting parameters, it is important to recognize that the actual operation space of MSCC systems is inherently multidimensional. In reality, a multitude of additional factors may influence the feasibility, stability, and robustness of MSCC experiments. Beyond the parameters considered here, aspects such as the temperature, defined gas composition (e.g. oxygen and carbon dioxide levels), medium viscosity, as well as further imaging parameters, such as the wavelength of the light, can affect both the physical microenvironment and the physiological response of the cultivated cells (see Figure 2c — blue area). These parameters often interact in complex, nonlinear ways, and even subtle variations can alter the local conditions experienced by single cells. Therefore, while simplified two-dimensional MWOs provide a valuable conceptual framework for understanding key dependencies, the multidimensional nature of microfluidic operation spaces must be kept in mind when interpreting results or transferring findings to other experimental settings.

Recent methodological advances may further accelerate the establishment of quantitative MWOs. Computational tools such as computational fluid dynamics (CFD) enable predictive modeling of flow, mass transfer, and concentration profiles, helping to fine-tune experimental parameters, as demonstrated by Westerwalbesloh et al. [54]. Beyond CFD, data-driven approaches such as machine-learning are emerging as particularly promising. These models can integrate experimental and simulated data to predict system behavior across a wide range of operating conditions and even under design modifications. For instance, Lashkaripour et al. [55], demonstrated how machine-learning-based modeling reduce experimental workload and accelerate the identification of viable operational regimes.

Ultimately, integration of DoE, CFD, and machine-learning approaches will enable more predictive and adaptive microfluidic experimentation, supporting systematic optimization of operational robustness and biological relevance in future MSCC designs [53–55].

Outlook

The MWO provides a conceptual and practical framework for linking experimental operation and biological performance in perfusion-based MSCC. Translating the bioprocess-window approach from conventional bioreactors to the microscale enables systematic definition of operational limits. Identifying key parameters such as FR and TPF, together with their system and experimental constraints, establishes feasibility, robustness, and biologically meaningful experimental conditions.

Future progress will shift the MWO from a qualitative to a quantitative framework. Mapping the multidimensional parameter space through experimental and computational tools, combining DoE strategies with CFD and machine-learning-based modeling, will allow *in silico* prediction of

suitable operating ranges. Such integration can drastically reduce experimental workload and guide the rational design of microfluidic systems and experiments.

In practice, quantitative MWOs could serve as a decision tool for selecting system operation, imaging parameters, and cultivation conditions, ensuring optimal biological insight at reduced experimental effort. Furthermore, MWOs defined for one strain or system may serve as transferable templates for related biological models or device generations, promoting standardization. In particular, the concept of MWOs, which was introduced here for perfusion-based MSCC, could be transferred to other microfluidic cultivation concepts, such as droplet-based microfluidics.

In the future, adopting the concept of quantitative MWOs to microfluidic cultivations could transform the field from empirical exploration into a predictive, data-driven discipline, advancing feasibility and robustness, accelerating development cycles of new designs, and moving the field toward rational, automated single-cell bioprocess engineering.

Conclusion

MWOs were introduced as a qualitative early-stage design tool for MSCC, enabling systematic evaluation of the interplay between flow conditions, nutrient supply, and imaging constraints. By mapping feasible operational spaces, this approach facilitates informed design decisions that balance physiological relevance, experimental stability, and measurement precision. The framework helps to identify limiting factors early in development, reducing costly iterations and improving reproducibility across microfluidic studies. Beyond providing design guidance, our analysis emphasizes the importance of integrating biological and engineering perspectives into microscale cultivation design. Considering the ongoing debate in this field, we trust that this study will serve as a foundation for future discussion and refinement of the ideas presented here.

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CRediT authorship contribution statement

Yannick Scholz: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Boris Yermakov:** Conceptualization, Writing – original draft, Writing – review & editing. **Alexander Grünberger:** Conceptualization, Investigation, Visualization, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no competing interests.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT 5 in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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