Trends in Biotechnology

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Review

Single cell technologies for monitoring protein secretion heterogeneity

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Cell-to-cell heterogeneity presents challenges across various fields, from biomedicine to bioproduction, where precise cellular responses are vital. While single cell technologies have significantly enhanced our understanding of population heterogeneity, the predominant focus has been on monitoring intracellular compounds. Recognizing the added complexity introduced by the secretion system, in this review, we first provide a systematic overview of the distinct steps necessary for driving protein secretion. We discuss the various sources of noise acting from the synthesized preprotein to the secretory protein released based on a Gram-positive cellular system as a model. We next explore the applicability of single cell technologies for monitoring protein secretion throughout these functional stages. We also emphasize the importance of applying these single cell technologies for monitoring protein secretion during bioproduction.

Highlights

Cell-to-cell heterogeneity in products secretion is a bottleneck for diverse applications in bioengineering, including controlled drug release, biomaterial assembly, and production of recombinant proteins in bioprocesses.

Experimental approaches aiming at characterizing cell-to-cell differences in gene expression and protein accumulation can be partially adapted to address heterogeneity in protein secretion.

Significant technological bottlenecks need to be addressed to establish an efficient single cell analysis pipeline.

Techniques designed for directing cell collective behavior (e.g., microfluidics or flow cytometry with feedback control) could be adapted for directing product secretion by cells.

Applying the single cell toolbox for the analysis of secretory proteins

Single cell analysis has contributed significantly to our understanding of the impact of cell population diversity and the development of appropriate control/mitigation strategies. However, most of the single cell-based technologies available are used for monitoring and controlling the accumulation/degradation of intracellular compounds (Figure 1A,B). While the intracellular production of a secretory protein can be monitored through single cell technologies (such as using a reporter strain where promoter activity is monitored through the production of a fluorescent protein), tracking the process of protein secretion poses a challenge due to the disconnection between the secretory protein and its originating secreting cell. Consequently, the analysis of secretory proteins is often limited to bulk measurements (i.e., supernatant of a cell population) and, thus, represents an averaged value of the given cell population (Figure 1C,D). This limitation results in a lack of knowledge regarding the emergence of population heterogeneities during the production of secretory proteins and the strategies required to control and mitigate them at the single cell level.

Accordingly, there is a pressing need for the development of related technologies in various fields, including biomedicine (e.g., drug release by live therapeutics [1]), biomaterials (e.g., for the release of amyloid and associated proteins as living glue [2]), and industrial biotechnology. In industrial and pharmaceutical biotechnology, a key challenge lies in gaining better control over cell population diversity to improve the production of secretory products, such as recombinant vaccines or enzymes. Given the increased biological complexity of a secretory pathway, we first systematically analyze the different functional stages required for driving protein secretion and discuss these as distinct sources of noise that can affect protein secretion at a single cell level. Our goal here is not to review the different protein secretion machineries in various cellular systems (i.e., Gram-negative and Gram-positive bacteria, as well as eukaryotic systems); for relevant references at this level, please refer to [3–8]. Considering the intricate protein secretion machineries in Gram-negative bacteria and the fact that Gram-positive bacteria are the preferred choice for the production of secretory proteins, we focus on a Gram-positive cellular system as a model.

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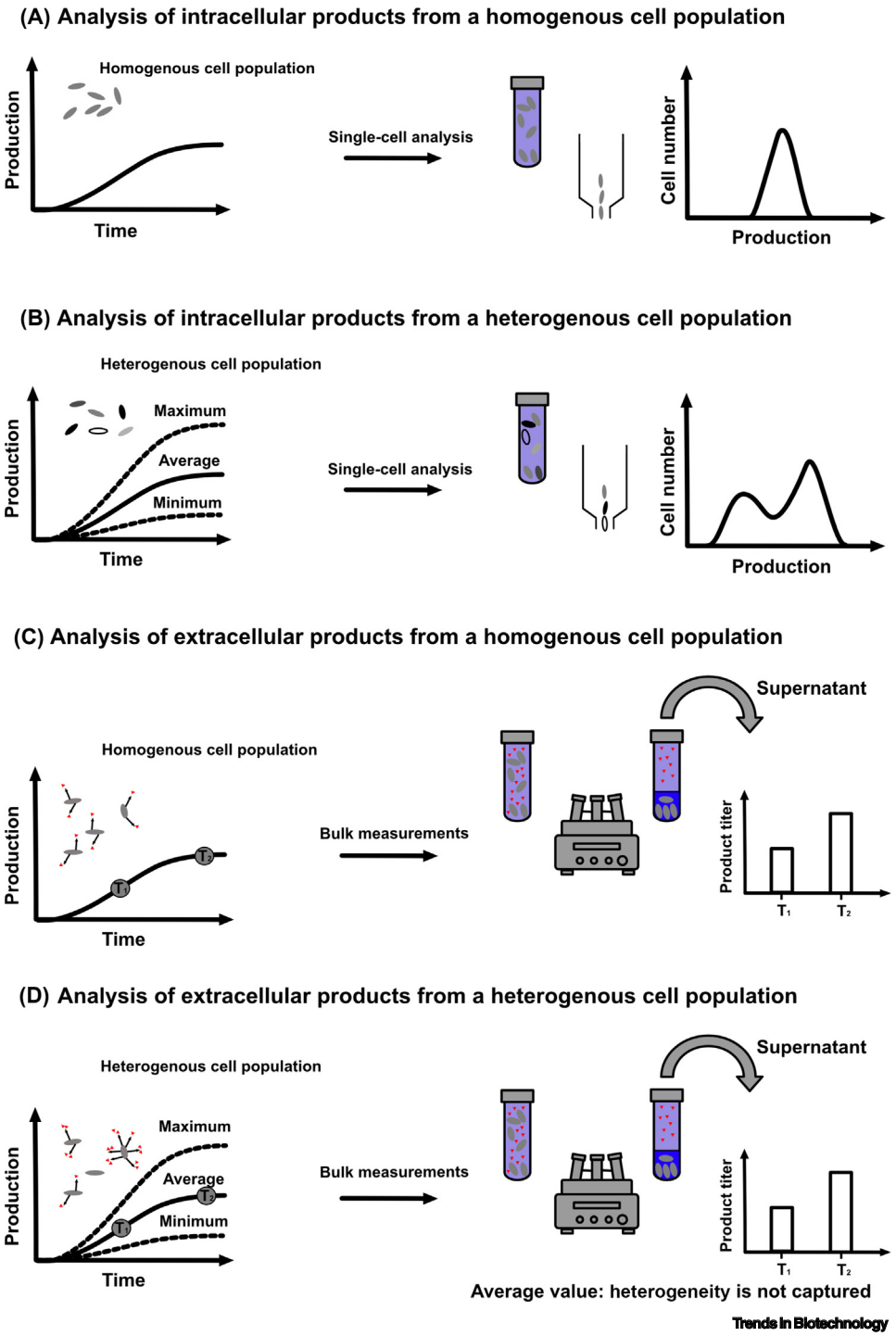

Figure 1. Analysis of cell population heterogeneity for intracellular and extracellular products. Single cell analysis (i.e., flow cytometry) is used to gain insights into the cell population heterogeneity of target intracellular products. In a homogenous cell population, the average production of the cell population reflects the productivity of each individual cell due to the uniform production of the target product (A). In a heterogeneous cell population, average values do not accurately represent the productivity of individual cells due to the presence of both low and high producers. The heterogenous productivity can be

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This approach enables us to explore the various functional stages of protein secretion and identify potential sources of noise in a more manageable context. Furthermore, we critically analyze current state-of-the-art technologies applied for characterizing and/or controlling protein secretion at the single cell level at the different functional stages. Finally, we discuss the challenges that need to be addressed to enable the successful translation of the single cell toolbox currently applied for intracellular compounds to secretory products. This will pave the way for establishing more efficient and dependable bioprocesses tailored for the production of secretory proteins, including industrial enzymes or biopharmaceuticals.

Different sources of noise affect protein secretion at the single cell level

Intracellular synthesis of the secretory preprotein

For both intracellular and extracellular proteins, the promoter element represents a key factor for the synthesis of the target product [9]. Initially, the expression of a target gene (i.e., encoding a secretory protein) is determined by the gene regulatory network (GRN) [9]. The promoter element constitutes the main feature of a GRN as it can promote the transcription of a target gene.

The promoter, located upstream of a gene on DNA, serves as the binding site for RNA polymerase, initiating the transcription of DNA into mRNA (Figure 2A). RNA polymerase comprises subunits (σ) that have a significant role in transcriptional regulation, as well as acting as repressors, transcriptional activators, and sigma-binding anti-sigma factors. Beyond transcriptional regulation, post-transcriptional systems impact mRNA stability and the rate of translation initiation by ribosomes. Despite a uniform cellular state within a population, stochastic effects, also referred to as intrinsic noise, can lead to variations in the timing and order of molecular processes governing transcription and translation in individual cells. Furthermore, molecular species involved in these processes (e.g., RNA polymerase, ribosomes, and transcription factors) are gene products themselves and, thus, can vary over time, which is referred to as extrinsic noise. The interplay of intrinsic and extrinsic noise results in cell-to-cell heterogeneity with respect to gene expression and, consequently, intracellular fluctuations in the production of a target protein. Thus, for a secretory protein, heterogeneity may already occur during preprotein synthesis, preceding the initiation of the protein secretion process (Figure 2A).

Recent studies highlighted that a significant factor contributing to cell population diversity is the fitness cost associated with activating the GRN responsible for phenotypic changes, such as transitioning from a non-producer to a producer state in the context of industrial biotechnology [10]. To produce bio-based compounds at a high level, a promoter element should ideally be strong and tunable [11]. Popular choices for driving the production of recombinant proteins are inducible (sugars and IPTG), constitutive or auto-inducible promoters (growth phase or stress specific) [9,12]. In general, for inducible systems that require active uptake of the inducer through transporter proteins, variations between cells often arise from fluctuations in the distribution of these transport proteins [13,14]**.** As an alternative, noninducible promoters can be utilized to control target gene expression. Extensive analysis has been conducted on a range of promoters,

quantified via single cell analysis (B). By contrast, when investigating population heterogeneity for secretory products, implementing single cell methods becomes more challenging, making cell-free analysis (supernatant) necessary. Bulk measurements (i.e., enzyme assay, ELISA, proteomics, etc.) yield only averaged values from the entire cell population, limiting insights into population heterogeneity. While the average value accurately represents the secretion level of a single cell in a homogeneous cell population (C), it loses this accuracy when individual cells exhibit varying secretion levels (D). Existing single cell methods used for intracellular products face challenges in their application to secretory products due to the disconnect between the secreted product and corresponding secreting cell. This prompts the question of how these tools can be adapted and utilized for monitoring population heterogeneity in the production of secretory products. Figure created using Affinity Designer.

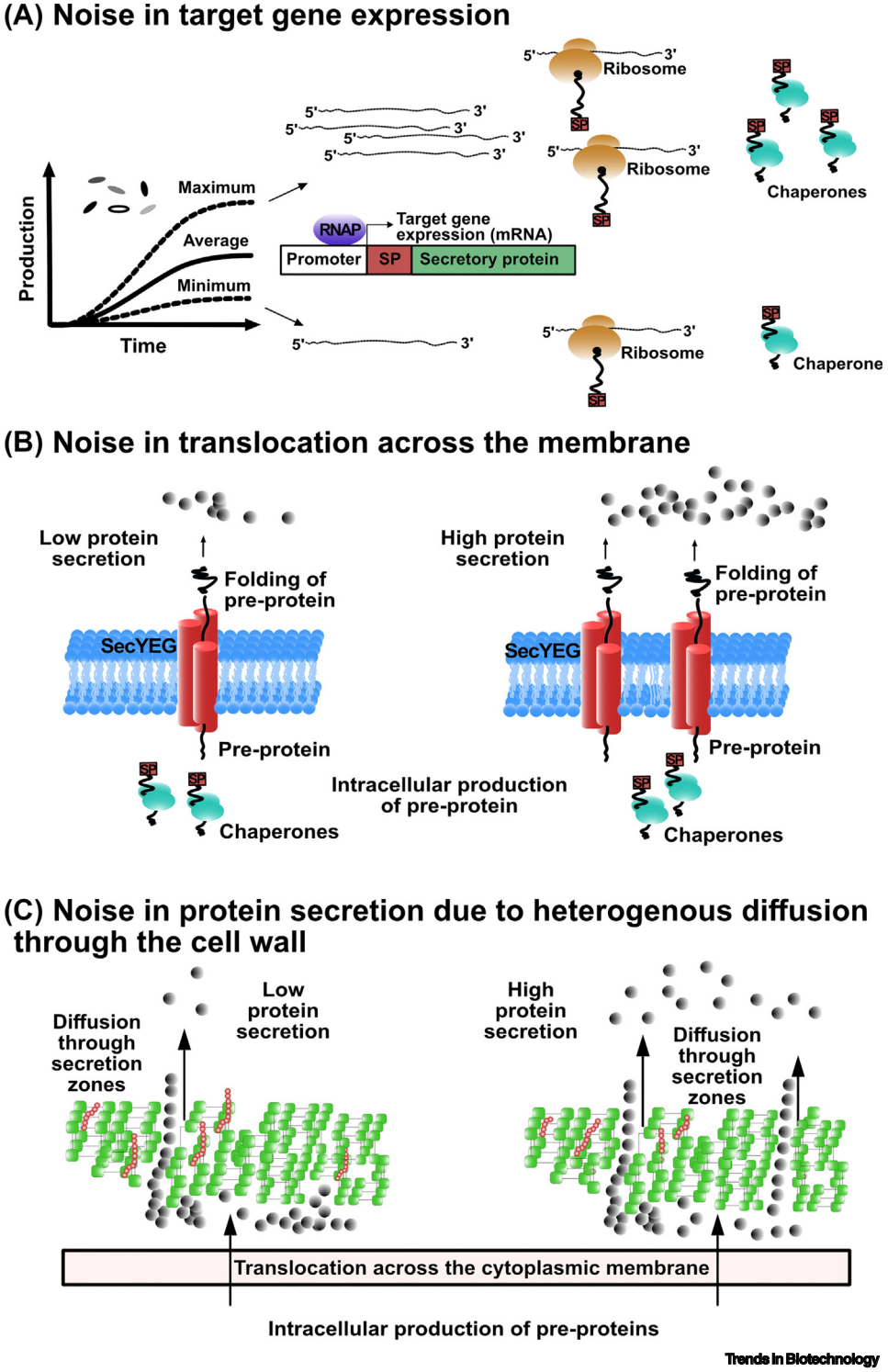

Figure 2. Heterogeneity in protein secretion induced at distinct functional stages during protein secretion and the methods available to capture population heterogeneity at the respective stages. During secretory protein

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particularly for common cell factories, such as *Escherichia coli* or *Bacillus subtilis*, down to the single cell level. Through this analysis, relatively homogeneous noninducible promoters have been identified [15,16]. The basic mechanisms behind biological noise (i.e., cell-to-cell variabilities at both the transcriptional and translational levels) are well known, and can now be more precisely characterized by approaches derived from statistical mechanics [17]. However, the resulting noise in mRNA and intracellular protein contents can be further propagated to the GRN and enhanced by the cell elongation and division processes, making its real function in cellular systems difficult to predict [18]. Accordingly, it is also difficult to assess whether homogenous systems are always the best solution for a given application. In a recent study, it was shown that retaining some cell-to-cell variability for the level of activation of burdensome gene circuits, such as the T7 expression vector used at the industrial level for the production of recombinant protein production, can provide stability to the whole population when cultivated in continuous mode [10]. Despite this observation, homogenizing the target gene expression in cell populations is still considered a reliable strategy to optimize both intracellular and extracellular bio-based product production [19]. However, the trade-off between growth and gene expression is important for the stabilization of cell populations [20], and maintaining a given degree of phenotypic heterogeneity can be advantageous in some cases (e.g., in continuous cultivation where a subpopulation of actively growing cells can rescue the whole population). Due to these uncertainties about the functionality exerted by biological noise on cell population, cell–machine interfaces have been developed for its mitigation in real-time during cultivation [21–24].

The secretion process adds an additional layer of complexity to the biological system, encompassing not only the initial synthesis of the pre-protein via transcription and translation, but also subsequent functional stages required for protein secretion. Next, we delve into the potential sources of noise arising at the different functional stages during protein secretion, elucidated using a single-membrane model system (Gram-positive bacteria).

Translocation across the cytoplasmic membrane

For the commercial production of secretory proteins, Gram-positive organisms, such as *B. subtilis*, are the preferred choice due to their single membrane system (Figure 2B), which streamlines the secretion process and offers advantages for large-scale protein production. Additionally, the Sec pathway is the preferred choice for commercial protein production because of its higher secretion capacity compared with the Tat pathway [25]. Thus, we focus here on a Gram-positive model system and the Sec-dependent secretion process of proteins (Box 1), exploring potential sources of noise.

Signal peptide (SP) engineering has become a major strategy for boosting the secretion of recombinant proteins, as most of the current SPs cannot meet commercial demands [26,27]. However, the usage of non-native SPs might cause inaccurate SP cleavage by the signal peptidases, which

production, variations in gene expression, such as noisy promoter elements, result in varying expression levels of the gene encoding the target secretory protein within individual cells of a population, leading to heterogeneous preprotein abundance for translocation (A). Following pre-protein synthesis, translocation across the membrane relies on exporter systems (e.g., Sec pathway), where the availability and activity of these systems determine the efficiency of protein translocation. Consequently, heterogeneity in exporter system availability or activity among individual cells leads to diverse translocation efficiencies. During translocation across the membrane, the pre-proteins are folded by the support of chaperones before their release into the environment (B). The cell wall is a thick, highly cross-linked copolymer, protecting the respective cytoplasmic membrane from the lethal effects of the high intracellular turgor. Before the final release into the environment, translocated and folded pre-proteins must traverse the cell wall through regions of lower density, referred to as secretion zones. Heterogeneity in secretion zones within the cell population can result in varying release efficiencies of folded secretory proteins, ultimately influencing protein secretion efficiency at the single cell level (C). Figure created using Affinity Designer. Abbreviations: RNAP, RNA polymerase; SP, signal peptide.

Box 1. The general secretion pathway

Following the synthesis of the pre-protein through transcription and translation, various components of the secretion machinery become essential to drive the process from the ribosome (where the pre-protein is translated) to the growth medium (resulting in the release of the secretory protein). The secretion-dependent (Sec) pathway is an evolutionarily conserved secretion system found in various organisms, including both Gram-positive and Gram-negative bacteria, as well as archaea and eukaryotes [71]. Often referred to as the general secretory pathway, it has a crucial role in translocating most secretory proteins across the cytoplasmic membrane [72–74]. The initiation of the targeting process for the secretory protein occurs shortly after the N terminus of the nascent protein emerges from the ribosomal exit channel [75], which involves the interaction between the nascent protein and ribosome-bound chaperons and targeting factors, such as the signal recognition particle (SRP) and SecA [75]. Sec-routed proteins typically feature a cleavable signal peptide (SP) at their N terminus, guiding them to the SecYEG membrane protein channel. SPs comprise 20–30 amino acids with a positively charged N-terminal region (N), a hydrophobic central region (H), followed by a short cleavage region (C), which is recognized by signal peptidases. This means that the secretion level of homologous or heterologous protein production is determined by the efficiency of the respective SP used to guide the protein secretion [26,27,75]. The Sec pathway facilitates secretion through a heterotrimeric integral membrane pore composed of SecY (the primary pore component with ten transmembrane domains), SecE, and SecG proteins. At this stage, the pre-protein is kept in an unfolded state by the action of different chaperones both in both Gram-positive and Gram-negative organisms. Subsequently, the nascent protein chains are directed to SecA, an ATPase associated with the SecYEG channel. The molecular motor SecA couples the binding and hydrolysis of ATP to facilitate the movement of the secretory protein across the SecYEG channel. Crystal structure and kinetic studies provide valuable insights into the binding mechanism of SP and the ATPase motor SecA and, based on this, binding and interaction of SP and Sec-pathway components were optimized to effectively boost heterologous protein secretion [29]. In this process, the N-terminal SP is removed from the target protein via signal peptidases, and the pre-protein undergoes folding with the help of chaperons and chaperone-like factors, which exclusively occurs at the extracytoplasmic side of the membrane [71].

can result in a heterogenous population of secreted proteins [28]. Besides the optimization of SPs, the Sec translocase itself has received increasing attention over the past few years as a target to refine the production of extracellular proteins [29]. In this context, the abundance and state of SecA emerge as central aspects that must be taken into consideration in the context of protein secretion heterogeneity (Box 2).

Another crucial aspect during protein secretion is the cellular folding capacity. An overwhelmed protein folding system results in an accumulation of misfolded proteins which are then degraded by so called quality control proteases [30,31], a stress phenomenon referred to as secretion stress (Box 3). Consequently, increasing chaperone expression is an efficient strategy to elevate protein secretion levels. For instance, the overexpression of *prsA* in *Bacillus* increased the secretion of alpha-amylases, recombinant protective antigen, and a protease [30].

Previous studies reported a secretion stress response manifesting heterogeneously in a subset of cells exhibiting a high protein secretion rate [11,32], a phenomenon that is known as secretion burnout [11,32,33]. This indicates that heterogeneity is a common occurrence during protein secretion,

Box 2. The impact of SecA on protein secretion heterogeneity

SecA is pivotal for the export process, functioning both as a receptor and a molecular motor; thus, it is indispensable for the translocation process across the inner membrane [75]. The dynamic localization of SecA is influenced by the production levels of secretory proteins and the presence of anionic phospholipids, such as phosphatidylglycerol and cardiolipin [36,76]. Notably, lipid and protein components are distributed diversly within the cytoplasmic membrane, as observed across various species [36,76]. For instance, the synthesis of phospholipids exhibits heterogeneity, and studies have demonstrated variations in the spatial distribution of phospholipids and glycolipids throughout the cell cycle [76]. In alignment with this, a dynamic and distinguishable, yet interconvertible diffusional population of SecA has been identified during protein secretion. This population comprises freely diffusing unbound SecA dimers, SecA proteins involved in a secretion complex, and a fraction diffusing through the cell or along the membrane while bound to a substrate [36,77]. Considering the pivotal role of SecA in driving protein secretion, the membrane composition and the cell cycle could significantly influence the efficiency of protein translocation across the membrane, potentially leading to heterogeneity in protein secretion. By contrast, heterogenous production of secretion components, such as SecA or SecDF, is less likely to contribute to protein secretion heterogeneity, as stated in a recent study where colocalization was investigated using fluorescently labelled SecA, SecDF, and AmyE molecules [36].

Box 3. Secretion stress

Secretion stress was first reported in the case of the high-level α-amylase production in *Bacillus subtilis*, but has also been observed in other organisms and for other types of secreted proteins.

In general, secretion stress involves the action of the following three systems: (i) the Sec secretion system; (ii) the CssR–CssS regulatory system; and (iii) the induction of the quality control proteases HtrA and HtrB.

CssR–CssS is a two-component regulatory system comprising the response regulator CssR and the membrane-embedded sensor kinase CssS. The sensor kinase CssS becomes activated upon accumulation of misfolded proteins at the outer cytoplasmic membrane and cell wall interspace or in response to heat stress. This results in activation of the response regulator CssR via phosphorylation [78,79], which induces the expression of genes encoding the membrane-attached quality control proteases HtrA and HtrB and the wall-bound protease WprA [78,80–82]. Quality control proteases are required to remove aggregated/misfolded proteins accumulated at the membrane–cell wall interface and, as such, avoid blockage of the Sec-translocase or cell wall growth sites [83].

emphasizing the need to account for it when engineering microbial systems for large-scale secretory compound production. However, the underlying causes of this variability remain uncertain and can arise from various factors. such as gene expression noise, inconsistent membrane translocation, or differing folding activities among individual cells on the *trans* side of the cytoplasmic membrane. Additionally, the final step of releasing and secreting the protein into the environment introduces another layer of complexity and potential source of heterogeneity, as described in the next section.

Passage through the cell wall

The cell wall can be considered as a porous, mesh-like hydrogel of peptidoglycan with a mean estimate of the effective pore (or mesh) size radius of 2.06 nm and 2.12 nm in *E. coli* and *B. subtilis*, respectively [34]. The meshwork accelerates the passage of globular hydrophilic molecules with an estimated size of 25 kDa [35]. It was recently hypothesized that the cell wall structure has areas of a lower meshwork density, allowing the passage of larger proteins [34]. This suggests that, during translocation across the cell membrane in Gram-positive organisms, passage through the cell wall occurs within specialized secretion zones rather than through equilibration across the cell wall [36] (Figure 2C). It was further demonstrated that the appearance of these secretion zones was highly heterogenous in an isogenic cell population of *B. subtilis* [36]. The authors observed that size and number of secretion zones increased as cells turned off cell wall synthesis upon entering the stationary phase [36]. This might be realized heterogeneously to supply substantial amounts of extracellular enzymes to the population while simultaneously just a minority of cells are going at risk of bursting due to internal turgor [36]. However, the authors observed that the fusion construct AmyE-mCherry, which served as the basis for visualizing secretion zones in single cells of *B. subtilis*, exhibited a pronounced membrane association and failed to distribute uniformly in the cytosol when the SP was lacking. Consequently, the inherent characteristics of the fusion construct may have influenced the secretion behavior, rendering it potentially unsuitable for investigating the concept of secretion zones. Further studies are needed for a more comprehensive understanding of the underlying mechanisms and the identification of actuators responsible for the heterogeneous formation of secretion zones during secretory protein production. This will pave the way toward the development of strategies to effectively control and mitigate protein secretion at the single cell level.

The primary challenge in addressing this need is the scarcity of tools for precisely quantifying products during the secretion process at the single cell level. This scarcity hampers a comprehensive understanding of protein secretion mechanisms and population heterogeneity during bioprocessing, limiting our ability to develop effective control and mitigation strategies. Accordingly, in the next section, we explore state-of-the-art single cell technologies for direct and accurate monitoring of secretory proteins at the single cell level throughout the secretion process.

Single cell technologies for monitoring protein secretion

Cytometric-based methods

Numerous single cell technologies have emerged to enable the quantitative exploration of phenotypic heterogeneity, encompassing single cell 'omics, such as transcriptomics and proteomics [37,38] as well as optical methods, including fluorescent stains and fluorescent reporters [39,40]. Optical-based single cell methods facilitate investigations and, thus, have made significant contributions to our understanding of cell population diversity and have a pivotal role in the development of automated online monitoring of physiological states during bioprocesses, a prerequisite for the development of appropriate control and mitigation strategies [10,41,42].

Single cell techniques, such as cytometric analysis, have predominantly been used for intracellular processes and products. For example, reporter strains can be engineered by inserting a gene encoding a fluorescent protein downstream of the corresponding promoter (Figure 3A). This allows the fluorescence signal to serve as an indicator of intracellular signal abundance, facilitating quantitative single cell analysis. However, while this method captures heterogeneity in promoter

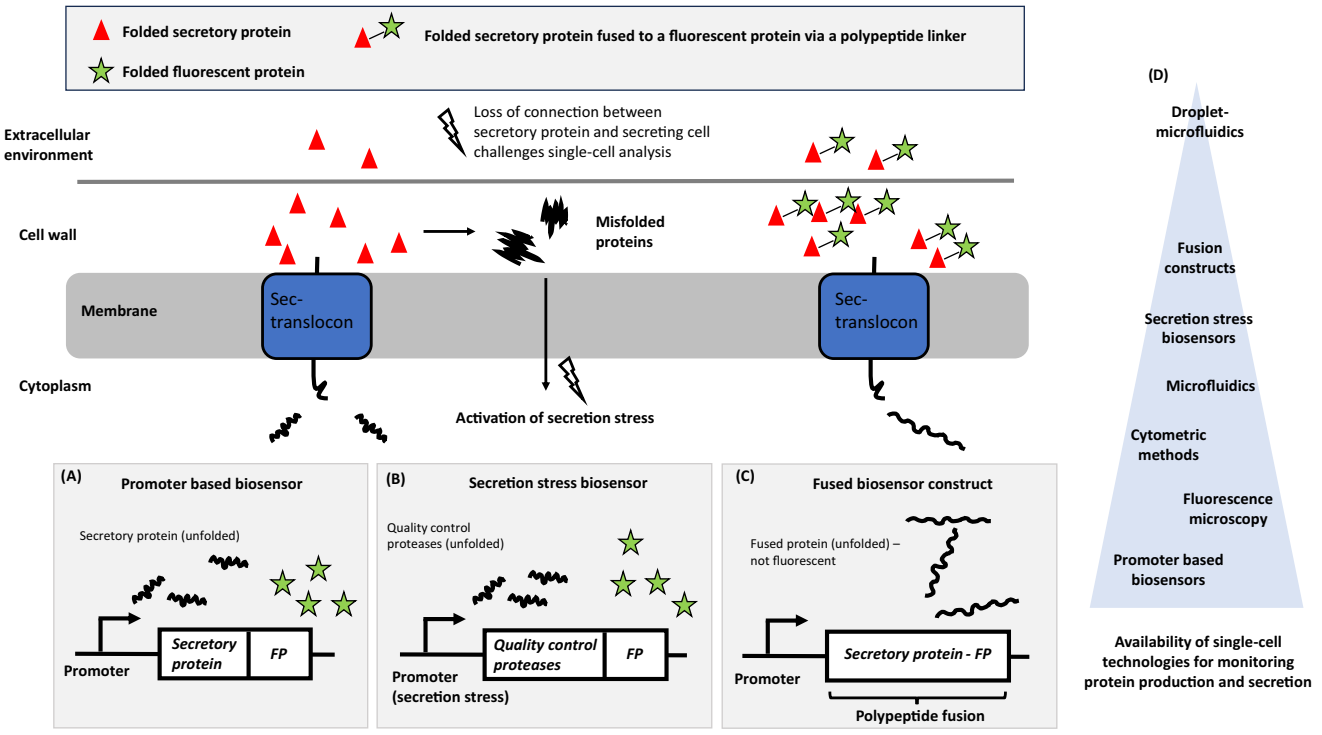

Figure 3. Single cell technologies available for monitoring the production of a secretory protein and the secretion process. In parallel with tracking population heterogeneity for intracellular products, the use of reporter strains employing promoter-based biosensors holds the potential to monitor secretory protein production within the cytoplasm. In this setup, the synthesis of the secretory protein is correlated with the production of a downstream reporter protein placed under the control of the cognate promoter element. Single cell technologies, such as cytometric methods or fluorescence microscopy, can be used to analyze these reporter strains, offering insights into population heterogeneity (A). However, this approach falls short in monitoring the entire protein secretion process, encompassing steps such as translocation across the membrane (via the Sec translocon) and passage through the cell wall before release into the environment. To address this limitation, a promoter-based biosensor responsive to secretion stress, triggered by the accumulation of misfolded proteins in the cell wall, can serve as an indicator of the quantity of proteins that have been translocated across the membrane (B). Additionally, secretory proteins can be fused to a fluorescent protein (FP) via a polypeptide linker. In this configuration, the fused construct is translocated across the membrane in a non-folded state (Sec-dependent protein secretion), resulting in the FP remaining nonfluorescent in the cytoplasm. However, it becomes fluorescent post translocation, enabling the use of single cell technologies, such as fluorescence microscopy and flow cytometry, to study population heterogeneity during protein secretion (C). However, tracking protein secretion at a single cell resolution becomes challenging once the secretory protein is released into the environment, leading to a loss of connection between the secretory protein and the corresponding secreting cell. Consequently, the availability of single cell technologies capable of monitoring protein secretion once the secretory protein has been released into the environment is limited to encapsulation methods, such as droplet microfluidics (D).

activity within the cell population, it does not provide insights into the diversity emerging during the secretion process. Capturing heterogeneity in protein secretion proves challenging due to the various functional stages involved in the process and the subsequent disconnection between the secretory protein and the corresponding secreting cell upon release into the environment. To overcome this challenge, reporter strains have been developed based on promoter elements involved in the secretion stress response (Figure 3B) [11,32,33,43]. Hereby, the fluorescence signal generated by these reporter systems can be used as an indicator of the number of misfolded proteins accumulating in the cell wall, which correlates with the quantity of proteins secreted by the respective cell [32,43].

In contrast to indirect monitoring approaches, labelling secretory proteins presents a strategy for directly quantifying the desired secretory product (Figure 3C and Table 1). Recently, a fusion construct was successfully developed by fusing a fluorescent protein via a polypeptide linker to a secretory protein [36]. The fused construct is designed with a single SP that is specific for the secretory protein. This arrangement enables the SP from the target protein to facilitate the efficient translocation of the fused protein construct across the cytoplasmic membrane (Figure 3C). For a Sec-dependent translocation, for instance, export across the membrane is required for proper folding into its natural state. Accordingly, all pre-proteins synthesized in the cytoplasm that are not translocated across the membrane should remain nonfluorescent, while the export of the fused biosensor construct allows monitoring of protein secretion at the single cell level due to the appearance of fluorescence [11,36]. This concept was used in a recent study to investigate the secretion behavior of the amylase AmyE in *B. subtilis* and *Bacillus licheniformis* by fusing the secretory protein via a polypeptide linker to the fluorescent protein mCherry [36]. When AmyE-mCherry was expressed as a polypeptide without the respective SP, eliminating translocation across the cell membrane, fluorescence dropped to background levels in *B. subtilis*. Intriguingly, in *B. licheniformis*, a notably higher fluorescence was observed in cells lacking the SP, indicating variation in the ability to fold and maintain the reporter protein in the cytoplasm between *B. subtilis* and *B. licheniformis* [36]. In light of recent findings, the application of fused biosensors must be validated for specific organisms, and appropriate controls (such as fused constructs without a SP) should be incorporated into the measurements to ensure reliability.

While direct labelling promises to quantify actual secretory proteins that have been translocated across the membrane, the release of the secretory protein into the environment severs the connection between the secreting cell and the labelled secretory compound. This challenges the single cell analysis of secretory proteins once released into the environment. To overcome these shortcomings, microfluidic-based methods can be used, as discussed in the next section (Figure 3D).

Microfluidic-based methods

To directly connect the production of an extracellular product released into the media with the corresponding secreting cells, it becomes imperative to isolate these cells into separate compartments, thus avoiding any potential signal disturbances that may arise. To achieve this, the utilization of miniaturized systems emerges as a highly advantageous approach. Not only does miniaturization reduce resource consumption, but it also facilitates the accumulation of secreted proteins at sufficiently high concentrations, enabling accurate analytical measurements [44]. The development of microfluidic technology has made the compartmentalization of single cells feasible [45–47]. Several techniques have been used in this regard, such as microwells [48,49], microdroplets [50], and hydrodynamic microtrap devices [51].

In droplet microfluidics, the encapsulation of single cells within discrete droplets, achieved by using a water-immiscible fluid, such as perfluorinated oil, is a fundamental technique. Here,

Table 1. Overview of different single cell technologies for monitoring heterogeneity in secretory protein production and secretion

Single cell technology	Detection method	Assay principle	Complexity	Resolution	Temporal resolution	Advantages	Disadvantages	Refs
Cytometry								
Flow cytometry, fluorescence microscopy	Fluorescence	Promoter/transcription factor-based biosensors	Low	Single cell	Medium	Simple designNatural secretion behavior of secretory protein	Fluorescence signal derives from cytoplasm (no direct quantification of protein secretion process)Slow temporal resolution due to slow degradation kinetics of fluorescent proteins accumulating in cytoplasm	[11,26,32,33]
		Labelling (fusion of fluorescent protein to secretory compound)	Medium	Single cell	Fast	Direct quantification of the target secretory compoundMonitoring during secretion process (signal in cell wall)	Low secretion efficiency of fluorescent proteinsFusion construct might affect natural behavior of secretory compound	[36]
Microfluidic								
Micro-/nanowell	Fluorescence	Labelling (fluorescent proteins, staining, tags)	Medium	A few single cells	Slow	Invasive labelling procedures necessary	Invasive labelling procedures necessaryTime-resolved measurements difficult	[48]
	Label-free	Surface plasmon resonance	High	A few single cells	Fast	High spatiotemporal resolution (real-time)No labelling procedures necessary	Complex set-up	[55,84]
Droplets	Fluorescence	Labelling (fluorogenic substrates)	Medium	A few single cells	Medium	Ultra-high-throughput (i.e., screens of enzyme libraries, signal peptide libraries)Simple analysis (fluorescence signal)	Fluorogenic substratesLong incubation time to accumulate a sufficient amount of the analyte	[85,86]
	Label-free	ESI-MS/MALDI-MS	High	A few single cells	Slow	Label-free analysis of secretory proteinsUltra-high-throughput screens of microbial communities at single cell resolution	Long incubation time to accumulate sufficient amount of secretory proteinsComplex experimental set-up for analysis	[56,58]

fluorophore-labelled substrates that are sensitive to degradation by the target secretory protein are commonly used (Table 1) [52,53]. As the substrate is degraded by the secreted protein, the fluorophore is liberated, resulting in an amplified fluorescence signal. Similar to fluorescence-activated cell sorting (FACS), these droplets can be subjected to analysis and sorting based on specific parameters, offering a controlled and efficient means of investigation [44,54]. This strategy allows for dynamic tracking and quantification of enzymatic activity as an indicator of the quantity and quality (enzymatic activity) of the secreted compound within the droplets, providing valuable insights into cellular processes and molecular interactions.

Label-free biosensing technologies have emerged over the past two decades to tackle the challenges in label-based assays, such as tedious labelling processes involving fluorescent dyes or complex handling/washing steps. In a recent study, a high-throughput and ultrasensitive nanoplasmonic biosensor integrated with microwell compartment arrays was applied for monitoring secretion at a single cell level without the need for tedious labelling methods [55]. The nanoplasmonic substrate utilizes gold nanohole arrays, the spectrum of which shifts highly sensitively in response to the localized refractive index change upon binding of analytes on its surface.

In a recent study, nanoliter droplet arrays had a key role in the noninvasive analysis of protein secretion in yeast [56]. Here, nanoliter droplets underwent a 24-h cultivation period followed by interfacing the nanoliter samples from the supernatant with matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS) for the precise detection of secreted proteins [56].

Combining droplet microfluidics with electrospray ionization MS (ESI-MS) has proven to be particularly valuable for investigating minuscule reactor volumes in a high-throughput manner [44,50,57]. Using this approach, it has been possible to monitor the biosynthesis of lysine secreted from as few as ten cells of *Corynebacterium glutamicum* [57]. However, the presence of surfactant in the ESI-MS analysis prevented the detection of biocatalytic products at the single cell level. To overcome this issue, Wink *et al*. developed a microfluidic platform using glass chips to generate droplets and fluorinated capillaries to store surfactant-free droplets [50]. This innovative approach allowed for the quantification of product levels in distinct droplets harboring only a single cell, overcoming previous limitations and enabling the determination of product formation rates for individual *Saccharomyces cerevisiae* cells [50].

The integration of droplet microfluidics with ESI-MS has found application in the analysis of antibody secretion from encapsulated bacteria. Maler *et al.* used a droplet-based microfluidic platform as an ultra-high-throughput screening system, leveraging the diversity of an entire microbial community in terms of its production of antimicrobial compounds [58]. The methodology involved loading droplets with single cells, followed by extended incubation for bacterial cell propagation and secretion and subsequent analysis via ESI-MS. The established approach enables the screening of diverse cell mixtures at the single cell level, presenting a promising avenue for identifying novel high-secretor candidates in antibiotic production [58]. This demonstrates the potential of integrating droplet microfluidics, cell imaging, and MS for studying cellular processes and product formation at the single cell level. However, while this approach is suitable for screening mutant libraries or microbial communities with respect to their protein secretion capacity, the extended incubation times (up to 25 h) required for cell propagation and product accumulation present a limitation for real-time monitoring of protein secretion during bioproduction.

Future challenges in controlling protein secretion at a single cell resolution for industrial biotechnology and other applications

Cell populations are able to naturally mitigate the universal trade-off between growth and gene expression (e.g., genes involved in secretion stress; Box 3) by simultaneously exhibiting different phenotypes within the same population [59,60]. This fundamental understanding of how cell populations exploit biological noise for functional purposes is key to enabling a series of applications. For example,

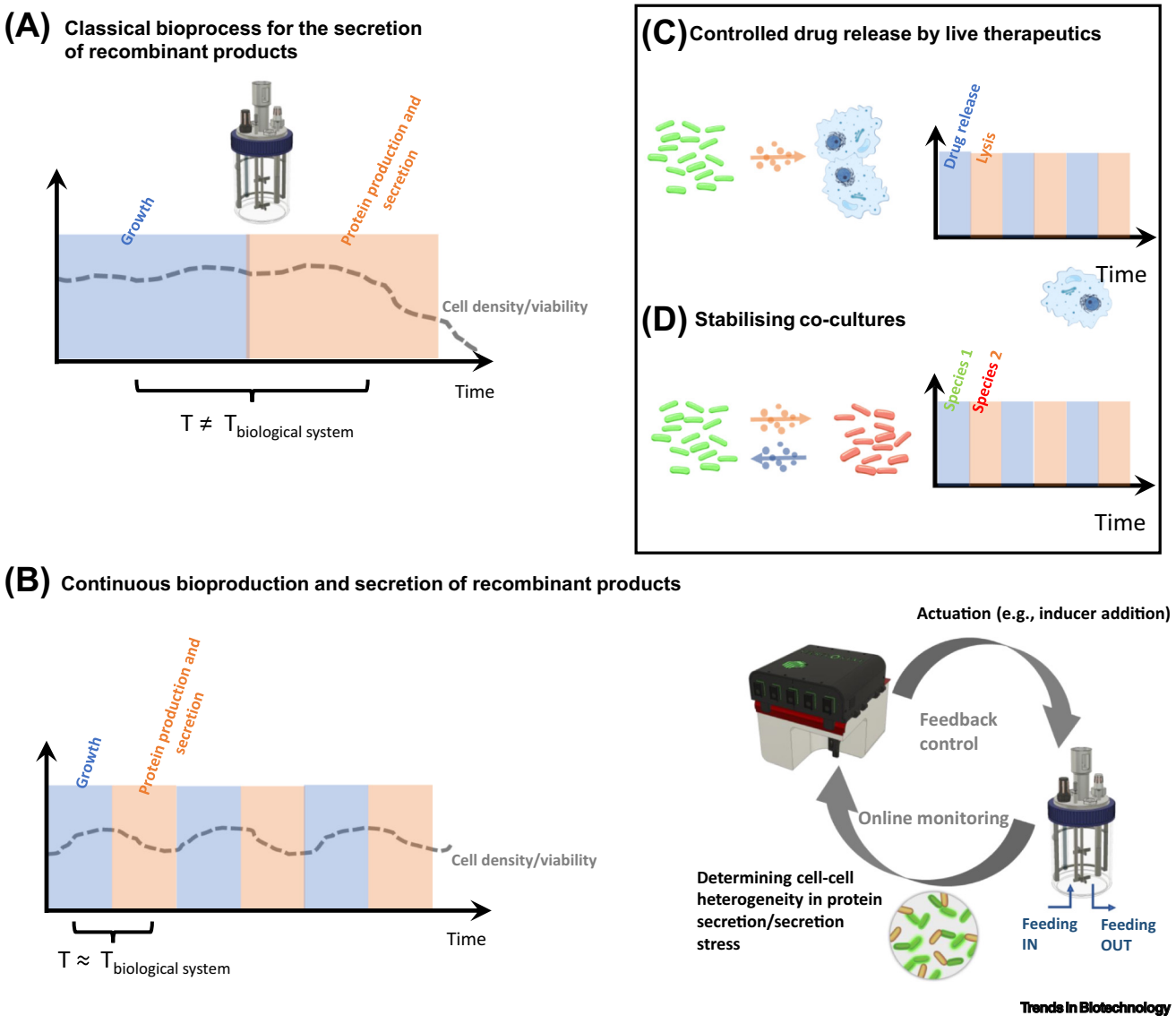

Figure 4. The cell–machine interface can be considered for actuating cell population and tailored protein secretion. (A) Classical operation for the microbial production of recombinant proteins. Two phases (i.e., growth and protein production/secretion) are successively considered during cultivation in bioreactors according to a timescale that is not compatible with the natural rhythm of the cell population (period associated with a biological cycle, represented by $T_{biological}$). Typically, the burden associated with secretion stress leads to the collapse of the population and the premature termination of the process. (B) Cultivation conducted with a cell–machine interface relying on automated flow cytometry and fluorescent reporter systems for monitoring protein secretion/secretion stress. Based on the response of the cell population itself (and its associated rhythm, represented by $T_{biological}$), the growth/secretion cycles can be controlled, which increases the robustness of the cell population. This technology can be used to ensure the transition towards continuous bioprocesses. This approach enables applications in other fields of research, such as (C) efficient in situ drug delivery to tumor cells by bacterial populations and (D) stabilization of co-cultures and characterization of the secretory pathways involved in the resulting microbial interactions.

recombinant protein synthesis is achieved by attempting to separate two phases, growth and gene expression, but on timescales that are not compatible with classical biological rhythms (Figure 4A). This frequently leads to system collapse after a few generations, impairing the development of more efficient bioprocesses, such as continuous bioproduction [20,22,61,62].

The advancement of single cell technologies (i.e., flow cytometry) combined with appropriate optical reporter systems has significantly improved the assessment of intracellular states of microbial cell populations at the single cell level [10,41]. This is key toward the development of multiscale digital models for both cell factory design and process optimization at industrial scales [39]. Based on automated flow cytometry, cell–machine interfaces have been developed in which both the external perturbation and monitoring of gene expression in living cells are completely automated [23]. By utilizing external perturbations automatically adjusted through cell–machine interfaces, such as repeated applications of specific chemical inducers at defined frequencies and amplitudes, intracellular responses (i.e., gene expression) can be dynamically coordinated [10,22]. These periodic stimulations lead to stabilized microbial populations in continuous cultures and match the timescales of cellular processes [10] (Figure 4B). While this strategy has proven effective for intracellular products, quantitatively monitoring protein secretion at the single cell level is more challenging due to the disconnection between the secreted compound and secreting cell. To address this challenge, a widely adopted approach involves using reporter systems to indirectly measure protein secretion, specifically by detecting the accumulation of misfolded proteins and their activation of the intracellular secretion stress response [32].

Recently, there has been substantial interest in the development and utilization of secretion stress reporter systems to enhance cell factory performance in secretory protein production [21,32,63]. The high interest in utilizing this reporter system for monitoring protein secretion stems from its straightforward design. It operates by generating an intracellular output signal, specifically the production of a fluorescent protein in the cytoplasm. This signal is activated in response to the accumulation of misfolded proteins in the cell wall. This design aligns seamlessly with established reporter systems for intracellular products and is fully compatible with contemporary single cell techniques, such as flow cytometry, FACS analysis, and fluorescence microscopy. This facilitates the development of automated control strategies in which the secretion stress level can be maintained at an optimal level [21,32,63]. By leveraging adaptation mechanisms favorable for secretion and avoiding detrimental effects, such as protein degradation via a real-time control strategy, a remarkable 70% improvement in protein secretion was achieved [32].

When using reporter systems to monitor protein secretion stress, it is crucial to recognize that fluorescence signals, although often interpreted as indicators of the secretory capacity of a cell, primarily signify the accumulation of misfolded proteins in the cell wall. Therefore, although these methods provide proxies for protein secretion and secretion capacity, they do not reflect the amount of properly folded and active proteins after their translocation across the cytoplasmic membrane. Industrial proteins, such as lipases, amylases, and proteases, serve as crucial components in laundry detergents and the bakery industry. To achieve successful production, the host organism must efficiently express and secrete these industrial enzymes in a biologically active form at a high rate [30,64].

Fusing fluorescent proteins directly to secretory proteins provides a valuable approach for the direct monitoring of proteins that undergo membrane translocation and proper folding. Recent studies utilizing fluorescent-labelled secretory proteins (i.e., AmyE-mCherry) have yielded profound insights into the oscillatory passage of molecules through secretion zones in the cell wall [36]. This phenomenon is marked by bursts of release followed by phases of re-accumulation

of proteins translocated across the membrane [36]. This highlights the potency of direct labelling as a powerful substitute for indirect reporter systems in real-time protein secretion monitoring. Yet, to the best of our knowledge, its application for online control and mitigation of protein secretion in microbial bioprocesses remains unexplored.

For a comprehensive analysis of protein secretion across multiple functional stages within a single cell, the development of combinatorial reporter systems will be essential. These systems should integrate promoter- or transcription factor-based biosensors (e.g., monitoring secretion stress or promoter activity for target gene expression) with direct labelling strategies (e.g., using a fluorophore fused to the target secretory protein). This approach will provide valuable insights into population heterogeneity at different functional stages, including target gene expression, membrane translocation, and cell wall passage.

While promising for single cell protein secretion monitoring, the aforementioned optical reporter systems pose challenges that need to be addressed before their widespread usage. First, not all fluorescent proteins may be secreted efficiently by the host strain and, thus, fail to become fluorescent or misfold during the secretion steps. In general, the secretion of fluorescent proteins is a challenging undertaking and there are only a few examples where fluorescent proteins have been successfully secreted in Gram-negative and Gram-positive organisms [36,65]. Second, the process of direct labelling of secretory proteins could interfere with their normal function, impacting their secretion dynamics and, as such, the production yields, solubility, or bioactivity of the target product. Accordingly, it is crucial to ensure that the fluorescent protein selected for labelling approaches does not compromise the accurate representation of protein secretion. To address this, extended research studies are required to identify fluorescent proteins suited for direct labelling of secretory proteins. Last, the additional metabolic requirements associated with the expression and utilization of optical reporters requires cellular resources and, thus, might alter the metabolism of the respective cell. Therefore, it is important to evaluate the trade-offs between the benefits gained from optical reporters and the potential metabolic costs they impose for industrial platform organisms used to produce secretory proteins at large scales. Considering the aforementioned challenges, there is a pressing need for the development of novel online analytical tools that facilitate noninvasive analysis of secretory proteins. Recent advancements in microfluidic-based technologies combined with MS make the accurate analysis of secretory proteins from single cells feasible [56–58].

The integration of microfluidic-based methods into automated online sampling bioreactor setups holds the potential to revolutionize the analysis of protein secretion at the single cell level. By leveraging the actual protein secretion levels of individual cells, it becomes possible to automatically adjust feeding rates and other process parameters. Attaining precise control facilitates the optimization of bioprocesses using real-time data, a crucial stride toward creating digital twins for bioprocesses that guide intelligent decision-making in biomanufacturing. This aligns with the Industry 4.0 concept, emphasizing digitalization and automation [39]. However, the intricate experimental configuration, the potentially disruptive nature of MS, and the extended incubation times required pose challenges, restricting the routine application of these technologies in automated process control.

As a result, these advanced single cell methods find greater suitability for fundamental research rather than for practical applications. To overcome these limitations and move toward a fully integrated system that permits real-time single cell-level monitoring of protein secretion during bioprocesses, further technological advancements are imperative. An alternative solution is the adoption of at-line lab-on-a-chip analysis, which facilitates sample delivery and processing, as well as feedback-controlled feeding within an at-line process, as opposed to a fully automated online control system.

Overall, by harnessing the power of single cell technologies such as microfluidic-based technologies, but also novel and powerful reporter systems, researchers can delve deeper into the functional stages of protein secretion and pave the way for improved strategies to optimize protein secretion processes. Continued advances in this field will enable comprehensive analysis of secretory proteins, foster the development of more efficient and controlled bioprocesses, and enable applications in other fields of research (Figure 4C,D). Indeed, protein secretion is one of the critical steps to be considered for delivering therapeutic compounds directly to the tumor microenvironment via specific secretion systems [1,66,67] (Figure 4C). In this case, it is particularly critical to control the secretion of the therapeutic proteins when cells are located within the tumor microenvironment. A similar approach could also be considered to investigate the mechanisms involved in the stabilization of co-cultures by providing a succession of metabolic niches compatibles for both species [68] (Figure 4D). Besides the important cross-feeding mechanisms that can determine the fate of the co-culture and relying on the nonspecific release of metabolites [69] or quorum-sensing molecules, more specific secretion systems can be used to adjust the co-culture composition through the release of, for example, bacteriocins [70].

Concluding remarks

Single cell analysis has greatly enhanced our understanding of cell population diversity and control strategies, primarily focusing on intracellular processes and compounds. However, the multistep secretion process for secretory proteins introduces additional complexity, creating challenges for the application of the current single cell toolbox designed for exploring intracellular processes. Unlike intracellular processes, this results in a knowledge gap regarding population heterogeneity during protein secretion and the development of effective control strategies. The combination of advanced single cell technologies, including microfluidic-based methods and suitable single cell reporter systems, holds significant promise for unravelling the intricacies of protein secretion processes at the single cell level and addressing this gap. These advancements not only facilitate a deeper understanding of population heterogeneity at various functional stages, but also offer opportunities for real-time customization of protein secretion. While challenges persist (see Outstanding questions), such as selecting appropriate fluorescent proteins and assessing their potential metabolic impacts (in the case of optical reporter systems) or effectively integrating microfluidic-based methods into existing bioreactor setups, ongoing research and technological progress will undoubtedly enhance the ability to monitor and control protein secretion at the single cell level in the future. These advances will have far-reaching implications, including improved bioproduction of secretory proteins and applications in fields such as targeted drug delivery and co-culture stabilization.

Outstanding questions

How can we analyze the secretion process at a single cell resolution?

Can the classical approach relying on the use of fluorescent reporter proteins be applied for the single cell analysis of the secretion process?

What is the contribution of secretion to global biological noise? What is the impact on cell population heterogeneity?

What are the critical steps for the transport of recombinant products outside cellular systems? What is the impact in term of metabolic burden?

Can the cell-to-cell heterogeneity in secretion be harnessed for enabling further applications, such as more continuous bioprocesses, the efficient delivery of drugs by living biotherapeutics, and the design of new biomaterials?

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Declaration of interests

None declared by authors.

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