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Optogenetics and biosensors set the stage for metabolic cybergenetics

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Cybergenetic systems use computer interfaces to enable feedback controls over biological processes in real time. The complex and dynamic nature of cellular metabolism makes cybergenetics attractive for controlling engineered metabolic pathways in microbial fermentations. Cybergenetics would not only create new avenues of research into cellular metabolism, it would also enable unprecedented strategies for pathway optimization and bioreactor operation and automation. Implementation of metabolic cybergenetics, however, will require new capabilities from actuators, biosensors, and control algorithms. The recent application of optogenetics in metabolic engineering, the expanding role of genetically encoded biosensors in strain development, and continued progress in control algorithms for biological processes suggest that this technology will become available in the not so distant future.

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Introduction

Cybergenetics is an emerging field that employs computer interfaces to control dynamic biological processes in real-time by monitoring key parameters and using feedback controls to establish specific objectives [1–5]. Recent advances in optogenetic modulators, genetically encoded biosensors, and control algorithms for cellular processes have prepared the field of metabolic engineering for the imminent application of cybergenetics. Using cybergenetic approaches to study or engineer cellular metabolism would expand the capabilities of basic research and biotechnology and help to develop new modalities of dynamic control for chemical production in microbial fermentations [6]. The systems we envision comprise a strain engineered with a biosynthetic pathway for chemical production and the three following cybergenetic elements: 1) genetically encoded optogenetic actuators that enable the control of engineered and/or endogenous metabolic pathways with light; 2) genetically encoded biosensors that report on the activity of the engineered pathway and/or the metabolic state of the cell; and 3) computer algorithms that integrate biosensor outputs to inform actuator inputs in closed-loop control systems to optimize chemical production (Figure 1).

Most cybergenetic demonstrations have been conducted in microfluidic devices or in small chemostat bioreactors suitable to study dynamic transitions between alternative steady or semi-steady states (e.g. tracking a time-varying ramp or sinusoidal reference function) [7–10]. Therefore, applying metabolic cybergenetics to the long non-continuous batch or semi-batch fermentations typically used for microbial chemical production will require new capabilities. In non-continuous experiments, actuators and biosensors must not only be reversible, but also have response rates (of activation and inactivation) fast enough to accurately induce effects and emit monitoring signals, respectively, within the timescales of the required metabolic shifts. Equally crucial is that the actuators and biosensors are functional throughout the fermentation despite changing conditions (e.g. growth phase, substrate and product concentrations, cell density, etc.). Finally, control algorithms must be able to handle inevitable delays and potential shifts during fermentations in the correlations between actuators and their triggered responses, or biosensors and their monitored parameters. Although many of these capabilities are not yet available and critical obstacles still need to be overcome, especially for industrial scale applications, the variety of existing optogenetic actuators, biosensors, and control algorithms for biological processes [3,7,11,12•,13,14••] gives us reason to believe that metabolic cybergenetics for microbial chemical production is on the horizon.

The actuators

Optogenetic modulators are in many ways ideal actuators for metabolic cybergenetics in non-continuous fermentations [11,15,16]. In contrast to the chemical inducers

Figure 1

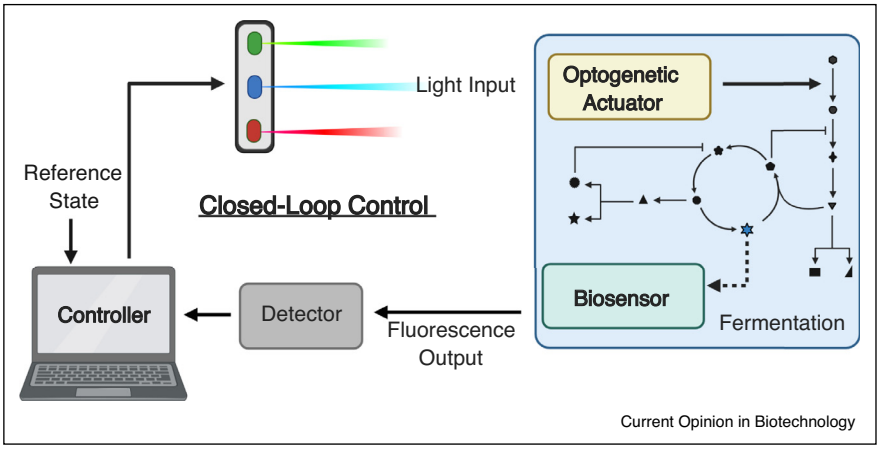


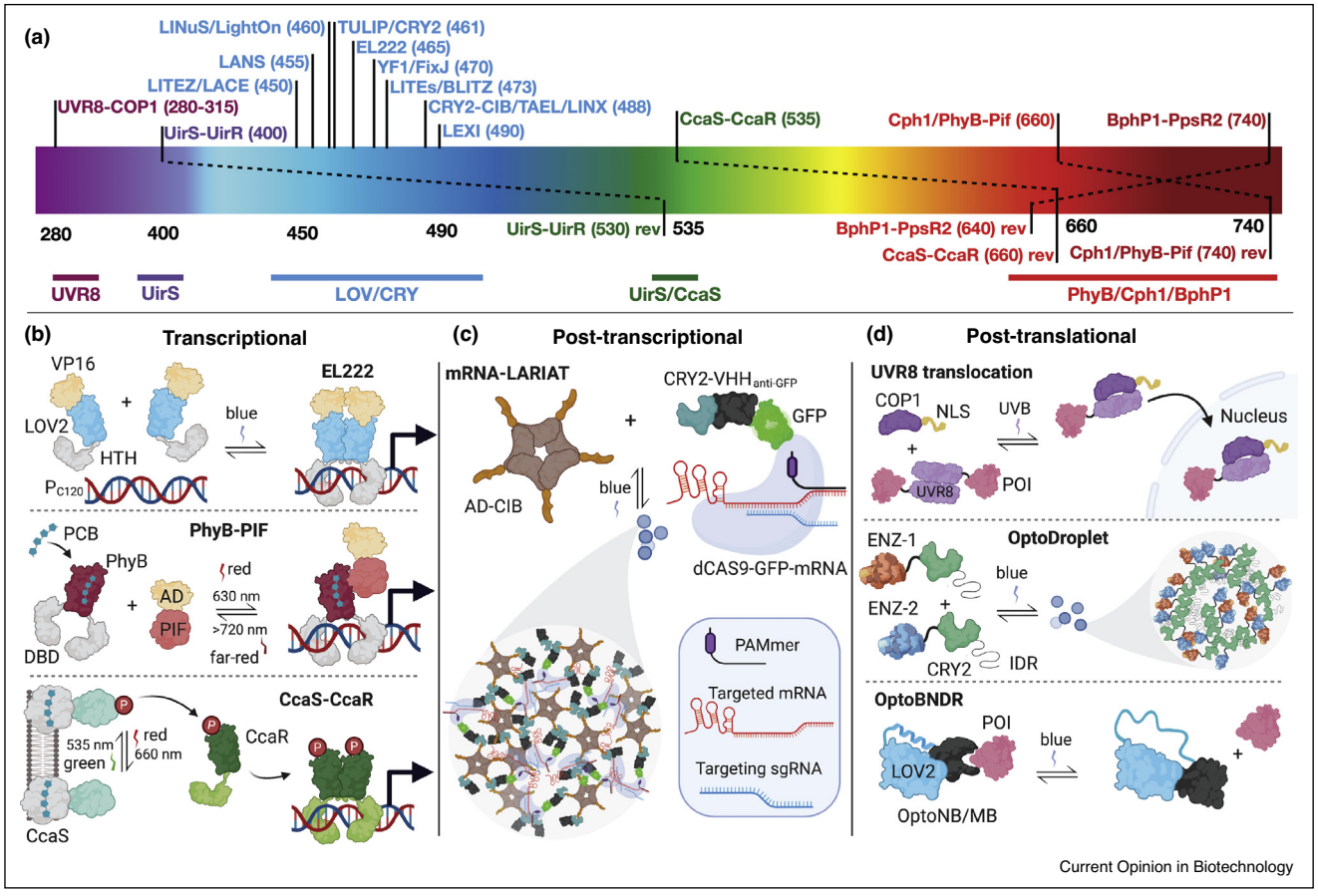
Diagram of a metabolic cybergenetic system. Optogenetic actuators read light inputs to control engineered metabolic pathway in microbial fermentations. Genetically encoded biosensors monitor the metabolic state by emitting a fluorescence output in response to concentrations of products, metabolites, or byproducts. A fluorescence detector feeds a controller, which compares this output to a reference value that optimizes metabolic performance, and corrects any deviation by changing the light inputs that feed the optogenetic actuators. This establishes a computer-assisted closed-loop control of metabolism. This figure was made using BioRender.

previously used in cybergenetics [2,3,17], light can be instantly applied or removed to control batch or semi-batch fermentations [18••]. Optogenetic actuators are also advantageous in the face of changing conditions as light inputs are reversible, highly tunable, operate independently of media composition, and can induce highly specific outcomes with minimal cellular side-effects [14••,18••,19•,20,21•]. Moreover, optogenetic systems can respond to a broad range of wavelengths (from infrared to ultraviolet, Figure 2a), and can operate at the transcriptional, post-transcriptional, or post-translational levels (Figure 2b–d). Optogenetic systems, therefore, offer many opportunities to develop rapid and reversible actuators for use in metabolic cybergenetics.

Optogenetic transcriptional controls respond to several wavelengths through varied molecular mechanisms [15,22] (see optobase.org). Their activation spectra span from UV to far red wavelengths [23] with distinct maximum excitation peaks (Figure 2a), although there is often overlap between the blue and red bandwidths (from 430 to 660 nm). In general, these regulators operate in either single-component or two-component systems (Figure 2b), both of which have been employed in cybergenetic applications. For example, a single-component system derived from the bacterial blue light-responsive transcription factor EL222 was used to establish a feed-back control system to study real-time gene transcription in single cells [20]. Separately, a transcription factor derived from light-triggered CRY2/CIB1 heterodimerization enabled the development of a closed-loop optogenetic compensation (CLOC) system which was employed to study feedback regulation and the yeast pheromone response [24••]. Additionally, the green and red light-responsive CcaS-CcaR two-component system was used to develop closed-loop control of GFP production and cellular growth rate in chemostats [9]. Some of these systems, in addition to the blue-responsive pDawn circuit, have also been applied in metabolic engineering for microbial chemical production [18••,19•] and metabolic flux studies [14••,21•]. Furthermore, because they act at the transcriptional level, these regulators can also be employed to develop optogenetic gene circuits [25–29], which can be used in metabolic engineering [18••,19•]. These gene circuits expand the capabilities of optogenetic controls, allowing them to regulate more complex processes [14••,18••,19•,21•]. For example, optogenetic circuits such as the yeast OptoINVRT can be used to invert the response to light [18••]. Although the versatility of transcriptional systems has made them the most widely used in metabolic engineering and cybergenetics, their response rates are inherently constrained by the rates of transcription and translation of the host organism.

By circumventing transcription, optogenetic systems that act at post-transcriptional level have the potential to serve as faster actuators. The recent discovery of the bacterial LOV photoreceptor PAL, which binds to short RNA aptamers preferentially in blue light, has enabled the development of optogenetic regulators of translation in bacteria and mammalian cells [30•]. A different strategy, called mRNA light-activated reversible inhibition by assembled trap (mRNA-LARIAT), provides translational control by sequestering mRNA in reversible blue light-assembled protein/RNA architectures. This reduces mRNA access to ribosomes, thus decreasing protein

Figure 2



Optogenetic systems with potential applications as actuators in metabolic cybergenetics. **(a)** The wavelengths to which optogenetic systems respond span from UV to far red (wavelength of peak activation shown in parentheses). Systems such as UirS-UirR, CcaS-CcaR, BphP1-PpsR2 and Cph1/PhyB-PIF are activated and inactivated at different wavelengths (indicated at the top and bottom of the spectrum respectively). Optogenetic systems work at the transcriptional **(b)** post-transcriptional **(c)** and post-translational **(d)** levels. **(b)** The single-component system EL222 fused to VP16 activation domain responds to blue light by dimerizing and binding to the P_{C120} promoter to induce transcription [18••]. PhyB-PIF heterodimers, which form and dissociate in red and far-red light respectively, can be fused to DNA-binding (DBD) and activation (AD) to make optogenetic transcriptional regulators [11]. This system requires Phycocyanobilin (PCB), which needs to be fed to or synthetically produced by the cells. The two-component system CcaS-CcaR, which also requires PCB, is activated by green light and inactivated by red light [26,28]. CcaS, a membrane protein, auto-phosphorylates and -dephosphorylates when stimulated with green or red light respectively. Phosphorylated CcaS phosphorylates CcaR, causing it to form homodimers, which bind DNA and induce transcription. **(c)** The mRNA light-activated reversible inhibition by assembled trap (mRNA-LARIAT) [31] combines three chimeras: (1) an assembly domain (AD) fused to CIB, (2) an anti-GFP VHH fused to CRY2 and (3) a dCas9-GFP fusion that sequesters mRNAs specified by a guide RNA (sgRNA). In the presence of blue light, CRY2 binds to CIB forming large protein-RNA clusters that inhibit translation of the sequestered mRNA. **(d)** UVR8 systems have been used to control nuclear translocation of proteins of interest (POI). When activated by UVB light, UVR8-POI fusions heterodimerize with its binding partner COP1 fused to a nuclear localization signal (NLS), inducing POI nuclear importation [32]. Optodroplets can cluster metabolic enzymes fused to CRY2 and intrinsically disordered protein regions (IDRs) into synthetic liquid membraneless organelles to direct metabolic flux [34]. Blue light activation of CRY2 oligomerization nucleates the liquid condensate formed by weak and transient interactions between the IDRs [77]. Optogenetic Binders (OptoBNDRs) [35,36] are fusions of the blue light-responsive AsLOV2 domain and customizable protein binders such as Nanobodies (OptoNB) or Monobodies (OptoMB), which reversibly bind to their cognate protein targets (POI) in response to blue light. Panels **(b)–(d)** were made using BioRender.

synthesis [31] (Figure 2c). While there are still relatively few optogenetic systems that operate post-transcriptionally, the examples that do exist demonstrate the potential of adapting this approach for cybergenetics.

Optogenetic systems that operate at the post-translational level bypass both transcription and translation, thereby providing the fastest rates of response and reversibility. These systems typically exploit light-triggered protein-protein interactions or the masking/unmasking of degradation, subcellular localization (Figure 2d), or protease cleavage sequences [11,15,16]. They respond to a wide variety of wavelengths, from PhyB/PIF-derived systems inactivated by infrared light to those derived from UVR8/

COP1 systems activated by ultraviolet light [32] (Figure 2a). Most systems, however, are derived from light-oxygen-voltage (LOV) domains (including iLID-SspB, magnets, LINuS, LANS, LEXY, LINX, and GLIMPSe) and, therefore, respond to blue light [11,15,16]. Additional blue-light responsive systems include CRY2/CIB heterodimers, CRY2/CRY2 oligomers, and blue-light dissociated PixELLs [11,15,16].

Post-translational optogenetic systems have enabled studies on complex dynamic systems. PhyB/PIF and CRY2/CIBN have been utilized to investigate cell signaling [11], and both iLID-SspB and CRY2/CIBN to study embryogenesis [33]. Additionally, light-controlled assembly of synthetic liquid membraneless organelles, mediated by CRY2 (Figure 2d) or PixELL systems, have been used to direct flux through branched metabolic pathways [34]. Optical binders (OptoBNDRS) [35,36], chimeras of AsLOV2 and nanobodies or monobodies (Figure 2d), which could be developed to reversibly bind any protein of interest, may further expand the capabilities of this approach. These early demonstrations suggest that cybergenetic systems may one day benefit from the fast-acting kinetics of post-translational optogenetic actuators.

The variety of optogenetic systems available raises the possibility of combining multiple actuators acting at different levels of control within a single strain. This would make it possible to benefit from the different advantages that each level has to offer (Table 1); for example, the rapid kinetics of post-translational controls with the robustness and economy of transcriptional regulators. Recently developed protein-based logic circuits [37•,38–40] could also be combined with optogenetic actuators to further expand the complexity of post-translational controls and their application in cybergenetics.

The ability to combine multiple optogenetic actuators responding to orthogonal inputs can also be useful for metabolic cybergenetics. To optimize chemical production, it is often necessary to balance the activity levels of enzymes in the biosynthetic pathway of interest and essential competing pathways. Therefore, having multiple actuators to independently control different sets of enzymes or pathways throughout non-continuous fermentations would significantly increase the capabilities of cybergenetic systems and the complexity of metabolic networks under control. The red/IR-responsive PhyB/PIF and UV receptors UVR8/COP1, have been combined to orthogonally control signaling pathways in mammalian cells [41]. Similarly, the phytochrome-based tools Cph1 (red/far red) and CcaS-CcaR (green/red) have been employed orthogonally to demonstrate bi-color control of gene expression [26,28]. Systems such as BpHP1/PpsR2 (far-red/red) and UirS-UirR (violet/green), could also be integrated in multichromatic systems as their range of activation wavelengths do not overlap with each other or with those of blue light-activated systems (Figure 2a). Other pairs with overlapping activating wavelength spectra may still be combined if at least one of them can be inactivated by a different non-overlapping wavelength. For example, while activation of Phy/PIF is maximal in the red spectrum, it is still sensitive to activation by blue light. However, because Phy/PIF is inactivated by IR, it could still be orthogonally used with a blue light system, using both blue and IR illumination to only activate the blue actuator. Overall, the diversity of optogenetic tools and gene circuit designs, as well as the potential to combine them [26,28,41] and use them in conjunction with biosensors [42] in the same cell, offer vast opportunities to develop effective actuators for metabolic cybergenetics.

The biosensors

Genetically encoded biosensors have been widely applied in metabolic engineering, enabling high throughput screens to identify better producing strains or hyperactive enzyme mutants, and to optimize metabolic pathways [43]. According to their mechanisms, biosensors can be grouped into four major classes (Figure 3): 1) ligand-binding-protein-fluorophore (LBPF) chimeras, including biosensors based on Förster (or fluorescence) resonance energy transfer (FRET) and circularly permuted fluorescent proteins (CPFPs); 2) RNA aptamers; 3) transcription factors (TFs); and 4) G-protein coupled receptors (GPCR). Each type of biosensor presents unique advantages and disadvantages (Table 1) in their potential application to metabolic cybergenetics.

Biosensors based on LBPF chimeras are fusions of natural or engineered ligand binding proteins and one or more fluorescent proteins (FPs). Conformational changes induced by ligand binding or environmental conditions (e.g. pH or oxygen level) shift the fluorescence profile of the chimera. In the case of FRET-based biosensors, the conformational change modifies the orientation or distance between pairs of compatible FPs, which allows for the transfer of energy from the excited reporter (donor) to its pair (acceptor), (Figure 3a) [44,45]. The output of FRET biosensors is thus a ratio of emissions from the FP pair, which reflects the intracellular ligand concentration. Although the dynamic range of FRET-based biosensors is usually limited, in most cases less than 3-fold [46], they can be relatively easy to design if a binding protein for the target molecule is available. In CPFP-based biosensors, ligand binding shifts the fused FP from an inactive to an active conformation (Figure 3b) [45]. Therefore, the total fluorescence emitted by these biosensors is proportional to the ligand concentration. The main advantage of LBPF chimera-based biosensors for metabolic cybergenetics is their ability to toggle between activated and inactivated states almost instantly. Several biosensors based on LBPF chimeras have been used to

Table 1

Advantages, disadvantages, and examples of cybergenetic components

Cybergenetic component	Classification	Sub-categories	Advantages	Disadvantages	Examples
Optogenetic actuators	Transcriptional	Single-Component	- Simple, robust, energetically economic - Increased versatility by enabling gene circuits	- Delayed by rates of transcription and translation - Restricted to UV and blue light	- EL222 [18••] - CRY2 [34,77,78]
		Heterodimers	- Sensitive to wide range of wavelengths, from UV to far red - Deep-penetration for systems responding to red wavelength - Increased versatility by enabling gene circuits	- Delayed by rates of transcription and translation - Some require external addition of cofactors - Differences in expression of each monomer may affect performance	- PhyB/PIF [11] - CRY2/CIBN [7,11] - BpHP1/PpsR2 [79]
		Two-Component systems	- Commonly used in cybergenetic demonstrations - Includes green light responsive systems - Increased versatility by enabling gene circuits	- Delayed by rates of transcription and translation - Restricted to bacteria - Reliance on phosphorylation cascades and multiple protein components - Increased potential for cross-talk - Some require external addition of cofactors	- CasS-CasR [9,10] - pDawn system [19•] - UirS-UirR [80]
	Post-Transcriptional	RNA/mRNA binding	- Faster than transcriptional systems	- Delayed by rate of translation - Current tools limited to blue light response - Can be energetically expensive for the cell	- PAL-aptamers [30•]
		mRNA sequestration	• Control over mRNA stability or accessibility		- mRNA-LARIAT [31]
	Post-Translational	Translocation	- Fastest rates of activation and reversibility by circumventing transcription and translation - Exploits subcellular organization/ localization for dynamic control	- Can be energetically expensive for the cell	- UVR8-COP1/NLS [32] - OptoDroplet [77] - iLID-SspB - PixELL [34,78] - OptoBNDRs [35,36]
		Phase separation			
		Protein-Protein interactions			

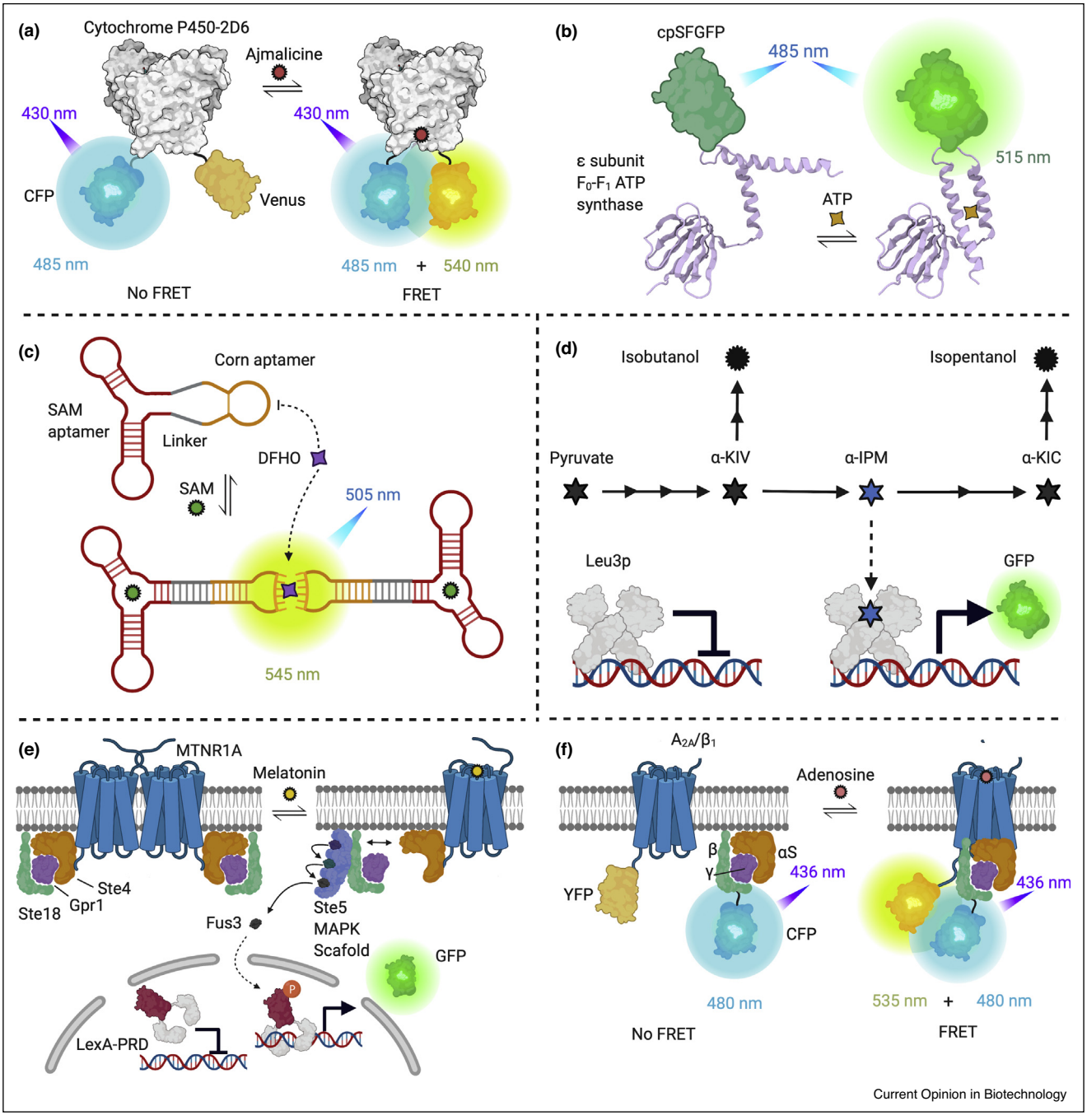
Table 1 (*Continued*)

Cybergenetic component	Classification	Sub-categories	Advantages	Disadvantages	Examples
Genetically encoded biosensors	Ligand-binding-protein-fluorophore	Förster (or fluorescence) resonance energy transfer (FRET) Circularly permuted fluorescent proteins	- Instant toggle between activated and inactivated states - Potentially easy to design and deploy	- Limited dynamic range (FRET) - Highly sensitive to intracellular variations	- Cytochrome-P450-based to measure Ajmalicine [46]. - Epsilon subunit of ATP synthase to measure ATP [48]
	RNA aptamers		- Systematic methods to obtain them (SELEX) - Instantaneous activation - High sensitivity and specificity - Limited intracellular cross-talk	- Irreversible ligand binding may limit or delay reversibility - Need of external fluorogenic additives	- Biosensors to measure SAM [54], Adenine [51] and Tyrosine [53]
	Transcription factors		- Most commonly used in metabolic engineering - Robust response - Energetically economic for the cell - Broad dynamic ranges - Highly tunable - May be linked to gene circuits, downstream events, or selectable markers	- Delayed by transcription, translation and reporter degradation/dilution - They can be difficult to obtain for desired molecules	- LTTR family-based biosensors to measure acyl-CoA [62], *cis,cis*-muconic acid [63] among others. Yeast - Leu3p-based biosensor to measure α-isopropylmalate [42]
	G-protein coupled receptors	Transcription-based biosensor FRET-based reporter	- GPCRs known to bind a large variety of chemicals - Highly sensitive due to signal amplification - Tunable and reprogrammable specificity	- Limited to yeast due to their reliance on endogenous signal cascades (not the case for FRET-based reporters)	- MTNR1A to sense melatonin [65] - The A2A GPCR-FRET based reporter for Adenosine sensing [66]

Table 1 (*Continued*)

Cybergenetic component	Classification	Sub-categories	Advantages	Disadvantages	Examples
Controllers	Proportional-integral feedback control (PI)		- Relatively simple to design and apply -Does not require a mathematical model -Able to reject constant perturbations	- Does not reject variable perturbations or systematically reduce errors over a period of time - Cannot handle time-varying references	- Control of GFP expression and cell growth [3,9]
	Model predictive control (MPC)		- It handles both constant and variable perturbations, also time-varying references - Predicts the output minimizing error overtime - Highly accurate	- Relies on a mathematical model to predict the output	- Control of GFP expression and cell growth [3,9] - Control of cell-cell variation and population structuring [10] - Control of yeast pheromone response [24••]
	'Bang-bang' controller		- Simple to design and deploy experimentally - Can handle both constant and variable perturbations - Especially suitable for toggle switches and control using forcing periods	- Difficulties to handle intrinsic delays	- Control of YFP using time-varying references in a chemostat [7] - Control of a genetic toggle switch to maintain cells in unstable equilibrium [69]
	Zero average dynamics (ZAD)		- Requires low number of input switches to track reference signals - It handles both constant and variable perturbations, also time-varying references	- May struggle to track fast reference signals	- Control of GFP expression at a set point or a time varying reference [3]
	Neural networks		- It handles both constant and variable perturbations including intrinsic systems delays - Data-based learning process that allows for improvement over time with additional data - May benefit from gray-box systems [71]	- Requires training with at least a limited data set - Needs a significant amount of data for accurate results - Computationally expensive - May depend on gray-box systems [71]	- Not yet demonstrated in cybergenetics

Figure 3



Major classes of genetically encoded biosensors with potential applications in metabolic cybergenetics. **(a,b)** Biosensors based on LBPF chimeras include FRET-based biosensors and circularly permuted fluorescent proteins. (a) Ajmalicine binding to a cytochrome P450-2D6-CFP-YFP chimera triggers a conformational change that brings the fused cyan fluorescent protein (CFP) and Venus into close proximity to induce FRET [46]. (b) A conformational change in the epsilon subunit of ATP synthase toggles a circularly permuted GFP from an inactive to an active state depending on ATP binding, allowing real-time monitoring of ATP levels in mammalian cells [48]. **(c)** RNA aptamer-based biosensors bind fluorogenic compounds that change in fluorescence upon ligand binding. The *S*-adenosylmethionine (SAM) binding-induced dimerization of the SAM-CORN aptamer creates a binding pocket for its fluorogenic ligand DFHO (3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime), emitting a fluorescent signal that allows the monitoring of SAM levels in living cells [54]. **(d)** Biosensors based on transcription factors that depend on ligand binding for activity. For example, Leu3p is a yeast transcriptional repressor in its apo state, but an activator when bound to the leucine precursor α-isopropylmalate. Leu3p has been used to construct TF-based biosensors for products derived from branched-chain amino acids, such as isobutanol and isopentanol (multiple steps in the pathway are indicated with multiple arrows) [42]. **(e,f)** Most GPCR-based biosensors harness endogenous signaling pathways to induce the expression of a reporter gene. For example (e), the human melatonin GPCR MTNR1A expressed in

monitor intermediate metabolites, such as pyruvate [47], cofactors like ATP [48] (Figure 3b), and final products, such as the monoterpenoid ajmalicine [46] (Figure 3a). Despite their high sensitivity and specificity, they require careful *in vivo* calibration as intracellular variations can greatly modify their output [49], which could pose challenges for their application in metabolic cybergenetics.

Biosensors based on RNA aptamers, short single stranded oligonucleotides, can sense proteins, cofactors, metabolites, and other molecules with high affinity and specificity [50,51]. Most commonly, these aptamers adopt alternative conformations, in which the analyte-bound conformation creates a binding site for an exogenous fluorophore, which, upon binding, emits a fluorescence signal [50]. A key advantage of these biosensors is that aptamers for a wide variety of ligands can be obtained with the well-established *in vitro* selection method SELEX [52]. These biosensors commonly attain nM to pM binding affinities to specific molecules and have dynamic ranges of 2-fold to 20-fold [48,53]. Other advantages of aptamers are their essentially instantaneous activation and their diminished cross-talk with other ligands compared to protein-based sensors. However, their tight binding is often practically irreversible, requiring aptamer degradation and fresh aptamer transcription to attain signal reversibility, adding delays. Another disadvantage of these biosensors is the need for fluorogenic additives, which can limit their use in large scale fermentations. This class of biosensors has been used to monitor intracellular concentration of *S*-adenosylmethionine (SAM) [54] (Figure 3c), tetracycline, adenine [51], and to select yeast strains with enhanced tyrosine production [53]. Despite their limitations, the versatility of this class of biosensor holds enormous potential for metabolic cybergenetic applications.

The biosensors most commonly used in metabolic engineering and synthetic biology are based on ligand-dependent TFs [12•,25,55]. These biosensors utilize transcriptional regulators that respond to the direct binding of specific molecules, including final products, precursors, or cofactors [56,57], to control the expression of a reporter (typically a FP, Figure 3d). Engineered variants with altered specificities can yield novel biosensors for different molecules [58••,59,60]. Bacterial small-molecule binding transcription factors from the lysR-type transcriptional regulator (LTTR) superfamily have been engineered to be functional in yeast [61], greatly expanding the potential to develop biosensors for different metabolites in eukaryotes. Many TF-based biosensors have been developed for metabolic engineering applications [56,57], including for the production of fatty acid-derived products (using the acyl-CoA-dependent bacterial TF FadR) [62], *cis*,*cis*-muconic acid (CCM) (using the CCM-dependent LTTR BenM) [61,63], and branched-chain amino acid-derived products (using the α-isopropylmalate-dependent fungal zinc-knuckle TF Leu3p) (Figure 3d) [42]. Biosensors for metabolic states have also been developed, such as for the NADH/NAD^+ ratio using the redox-responsive bacterial Rex TF [64]. While TF-based biosensors tend to have high dynamic ranges, the inevitable delays in their response and reversibility caused by the transcription, translation, and degradation/dilution of reporters raise concerns about their effectiveness in metabolic cybergenetics. Nevertheless, their robustness and established impact in metabolic engineering make a strong case for exploring their utility in this emerging field.

Biosensors based on GPCRs specialize in sensing specific extracellular molecules. They typically exploit GPCRs heterologously expressed in yeast, designed to trigger its mating signaling pathway to induce transcription of a reporter (usually a FP). For example, the human MTNR1A and A2BR GPCRs have been employed to sense melatonin (Figure 3e) and adenosine, respectively [65]. GPCRs have also been used to develop FRET-based reporters, which have the potential to be used as biosensors for metabolic engineering and cybergenetics (Figure 3f). It is expected that these biosensors would show enhanced response and reversibility rates as they would circumvent reporter transcription, translation, and degradation [66]. Key advantages of this class of biosensor include the large and growing number of GPCRs identified for a broad range of chemicals and the sensitivity obtained by signal cascade amplification. Additionally, they can be rationally tuned and reprogrammed to monitor a variety of molecules [65]. While their reliance on endogenous signaling pathways restricts their applicability in other host organisms, their many advantages (Table 1) raise the prospects of using this class of biosensor for metabolic cybergenetics in yeast.

The features that have made biosensors powerful tools for metabolic engineering are unlikely to be sufficient for their successful implementation in metabolic cybergenetics. In addition to canonical considerations such as sensitivity, dynamic range, and maximum output [65], biosensors applied to metabolic cybergenetics must also

(Figure 3 Legend Continued) yeast can be made to recruit Ste5 upon ligand biding, which triggers a phosphorylation cascade that leads to nuclear translocation of Fus3, activation of the LexA-PRD transcription factor upon phosphorylation, and expression of GFP [65]. (f) The GPCR-based biosensor for adenosine attracts the βγ subunits upon ligand binding, which brings fused YFP and CFP into close proximity, resulting in an increase in the YFP/CFP fluorescence ratio [66]. This figure was made using BioRender.

be reversible, show fast activation/inactivation kinetics, and have the ability to accurately monitor analyte concentrations throughout long non-continuous fermentations, including at high cell densities where fluorescence quenching is a concern. However, advances in de-novo biosensor design [67], novel biosensors to measure metabolites in real-time for pathway optimization and monitoring [68], and the use of biosensors in combination with gene logic circuits to control chemical production in real-time [58••] foreshadow the feasibility of using genetically encoded biosensors for metabolic cybergenetics.

The controllers

Equally important to the success of metabolic cybergenetics is the implementation of effective control algorithms. Cybergenetic studies have used different algorithms to establish feedback controls over a range of biological processes. Proportional-integral (PI) feedback control (which corrects set point deviations by measuring the current error and its time integral) and model predictive control (MPC, which utilizes mathematical models of the system to predict the output that will minimize the error over a time period) have been used to control GFP expression and growth rate [3,9,10,24••]. PI controllers have the advantage of being relatively simple and not requiring a mathematical model of the system. Additionally, they are effective at controlling systems with constant setpoints and rejecting constant perturbations. They fail, however, to adequately control systems with time-varying reference points or rejecting variable perturbations, for which MPC algorithms are much more effective [9]. Although MPC algorithms require at least a crude mathematical model of the system, they are superior for handling delays such as those caused by transcription or translation [24••].

Other controllers have proven to be useful for cybergenetics. The 'bang-bang' controller, which applies maximal or minimal control outputs depending on the sign of the deviation from setpoint, and zero average dynamics (ZAD), an approach derived from sliding control techniques, have been employed to control protein concentrations at fixed and time-varying references [3,7]. The 'bang-bang' controller has also been used to maintain a toggle switch in unstable equilibrium for extended periods of time, aided by a deterministic model of pseudo-reactions representing reporter transcription, translation, and degradation/dilution [69]. While a PI controller was able to maintain only a single cell away from the stable states of the toggle switch, the 'bang-bang' controller proved effective at also maintaining multiple cells at unstable equilibrium. This was done using periodic forcing [69], for which optogenetics is ideally suited [18••].

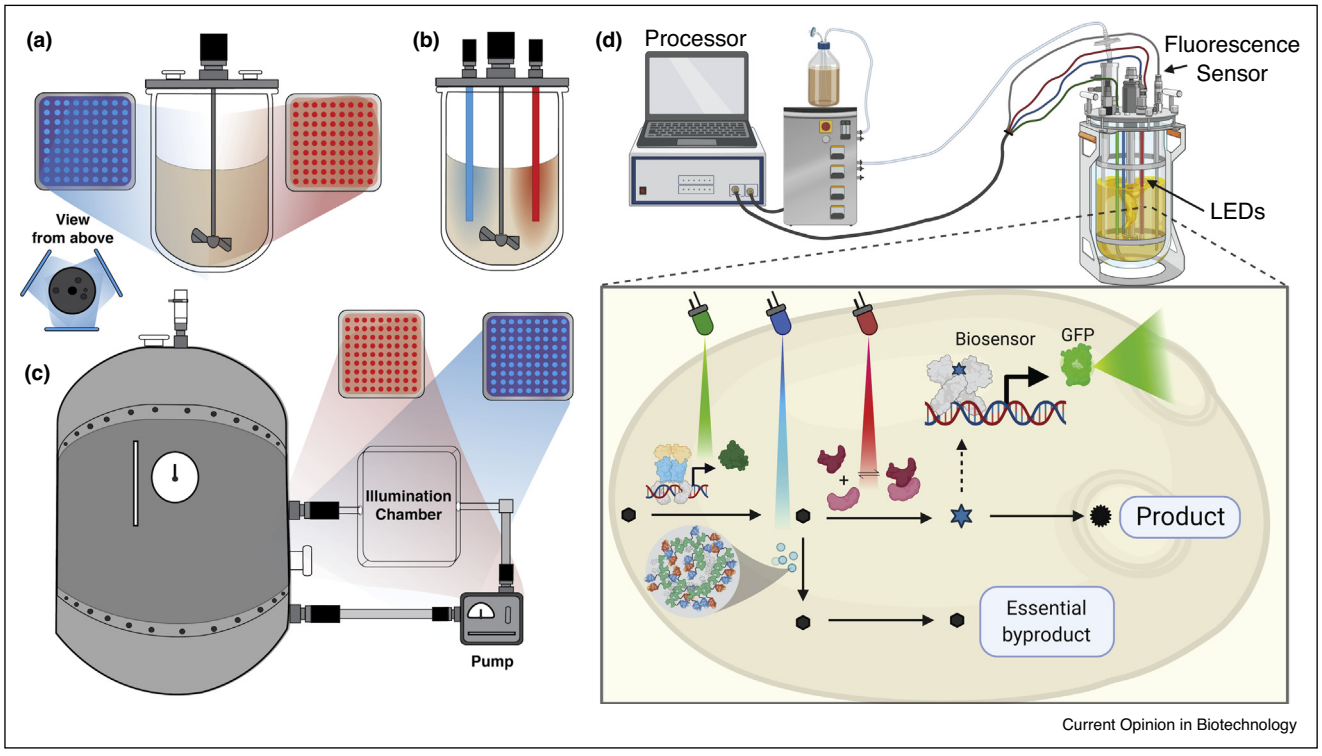
The ability of some of these algorithms (e.g. MPC and 'bang-bang') to track time-varying references and reject dynamic perturbations makes them particularly attractive to control complex dynamic systems. The recent development of an integral feedback controller that rejects noise as well as external and internal perturbations for robust and perfect adaptation, is another important step in the right direction [70••]. Alternative approaches, in cases where system delays are insurmountable, may use controls guided by neural networks trained with limited data sets, enabled by 'gray box' models that incorporate physical knowledge of the system [71]. This critical data may be acquired from high through-put experiments such as those using 96-well *optoPlates* [72]. Although none of these algorithms have yet been tested in large batch or semi-batch fermentations, their capabilities (Table 1) to efficiently and accurately handle delays, varying references, and dynamic perturbations open the door to their application in metabolic cybergenetics.

The equipment: closing the loop

Key to realizing the goals of metabolic cybergenetics is the development of proper equipment. Most cybergenetic demonstrations have been done in low-volume continuous systems (e.g. microfluidic devices or small chemostats) [8–10,24••] or relatively short-lived experiments [73]. This facilitates the use of light stimuli or even chemical inducers as inputs for actuators, given that volumes are small, there are no impediments to light penetration, and flexible experimental time-lengths can be extended as necessary. Implementing metabolic cybergenetics in bioreactors for microbial chemical production, however, will require different infrastructure because of the high cell densities they typically achieve and their non-continuous operation.

While fluorescence may be continuously monitored in bioreactors using commercial probes [74] or sample streams for flow cytometry [9], using optogenetic actuators can be problematic as light penetration in large fermentations will likely be limiting. Fermentations at cell densities of up to at least 50 OD_{600} in bioreactors of up to at least 5 L can be controlled with light by illuminating well-mixed cultures with LED panels through the walls of a glass vessel [18••] (Figure 4a). It is conceivable that a similar approach could be employed in larger industrial transparent plastic bioreactors [75]. Alternatively, many photobioreactors designed for photosynthetic organisms [76] could be repurposed, including several with submerged LEDs (Figure 4b). Another possibility is recirculating the fermentation broth through constantly illuminated transparent chambers, sometimes used for photoactivation of chemical reactions, the flow rate of which would set the frequency of forcing periods (Figure 4c). Despite the uncertainty of how metabolic cybergenetics may be implemented in industrial settings, its feasibility in lab-scale bioreactors is more predictable, which would open new research avenues in metabolic dynamics to better understand and optimize metabolic pathways (Figure 4d).

Figure 4



Strategies to illuminate bioreactors and general experimental equipment for metabolic cybergenetics. **(a)** Top-view and side-view of a transparent bioreactor illuminated by externally placed LED panels. **(b)** Side-view of a bioreactor illuminated by submerged LEDs. **(c)** Side-view of a large and opaque bioreactor illuminated by re-circulating flow through a transparent illumination chamber. **(d)** General equipment of a metabolic cybergenetic system, including a microbial strain engineered with a metabolic pathway for chemical production, orthogonal optogenetic actuators responsive to red, green, and blue wavelengths that regulate the activity of specific enzymes in the engineered pathway, and a genetically encoded biosensor that monitors the activity of said pathway. The fluorescence signals emitted by the biosensor are captured by a probe and processed in a computer, which uses a feed-back control algorithm to inform the outputs of LEDs in order to track a specific signal reference that maximizes chemical production. Panel **(d)** was made using BioRender.

Conclusion

Metabolic cybergenetics could transform the way we study metabolic dynamics, optimize engineered pathways, or even design and operate bioreactors for microbial chemical production. Although these strategies have tremendous potential, many challenges still lie ahead before researchers can practically implement metabolic cybergenetic approaches. These challenges include developing actuators and biosensors that are rapid, reversible, and functional throughout long non-continuous fermentations, as well as control algorithms that can handle inevitable delays and potential drifts in actuator/biosensor correlations to their cellular functions. Overcoming light penetration limitations will also be important for industrial applications. Nevertheless, the recent application of optogenetics to metabolic engineering, the expanding capabilities of genetically encoded biosensors, and ongoing efforts to develop controllers for increasingly complex biological processes have set the stage for the coming of metabolic cybergenetics.

Conflicts of interest statement

Nothing declared.

CRediT authorship contribution statement

César Carrasco-López: Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Sergio A García-Echauri:** Conceptualization, Writing - original draft, Writing - review & editing. **Therese Kichuk:** Conceptualization, Writing - review & editing. **José L Avalos:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

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