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Engineering living therapeutics and diagnostics: A new frontier in human health

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Abstract

Traditional therapeutics aim to diagnose, treat, and cure diseases through various synthetic and natural approaches. The emerging field of engineered living therapeutics (ELTs) genetically functionalizes living cells to alter the paradigm of designed solutions. In this review, we focus on ELTs derived from microbial cell scaffolds. We propose three synergistic modalities for the rational design of ELTs: first, use of regulatory operations to regulate genetic expression; second, integration of alternative biosensing inputs for directed application; third, choice of microbial chassis to deliver solutions. We highlight the challenges and future opportunities within each group and conclude by providing a prospective outlook for ELTs.

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Introduction

Recent trends in medicine have demonstrated a shift away from synthetic small molecule treatment and towards living therapeutics [1,2]. A living therapeutic uses a cell scaffold to diagnose, treat, or cure a disease. This approach is less expensive, reduces off-target effects, and localizes more effectively to the destination [3]. Unlocking the next generation of living therapeutics will require optimized, predictable, and programmable delivery to combat a myriad of ailments. Synthetic biology accomplishes this objective by integrating genetic circuits into a microbial chassis to create an engineered living therapeutic (ELT). ELTs can be created by modularly deconstructing the genetic framework into three modules: regulatory operations, biosensing modalities, and microbial cell chassis. This review highlights recent advances and emerging trends in each of these approaches (Table 1) and motivates the need to iterate amongst the three for the development of highly specialized and targeted engineered living therapeutics (Figure 1).

Regulatory operations

ELTs process a given input signal using regulatory operations to provide a desired cellular response. A primary operation is logical decision making, where transcriptional regulators control the expression of a genetic output via conformational changes of binding their cognate inducer input. All 16 Boolean logical gates can be genetically programmed *a priori* using this framework (Figure 2a) [4,5]. Recently, scientists have enhanced regulation pathways that respond to disease-relevant biomarkers for diagnostic purposes. Vaaben et al. characterized regulatory mechanisms in probiotic strain *Escherichia coli* Nissle 1917 (EcN) activated by gastrointestinal biomolecules, including primary and secondary bile acids, oxygen, lactate, caffeine, pH, and acetoacetate. Each biochemical input induced a corresponding transcriptional regulator to express a fluorescence protein marker. These distinct logical BUFFER operations validated the effect of non-standard signaling conditions under gut-like conditions, using *Caenorhabditis elegans* [6]. Similarly, Harimoto et al. engineered a BUFFER gate *in vivo* that responds to the synthetic inducer isopropyl-b-D-thiogalactopyranoside (IPTG) [7]. These two studies demonstrate the potential for ELTs as continuous biosensors or therapeutic payload delivery mechanisms.

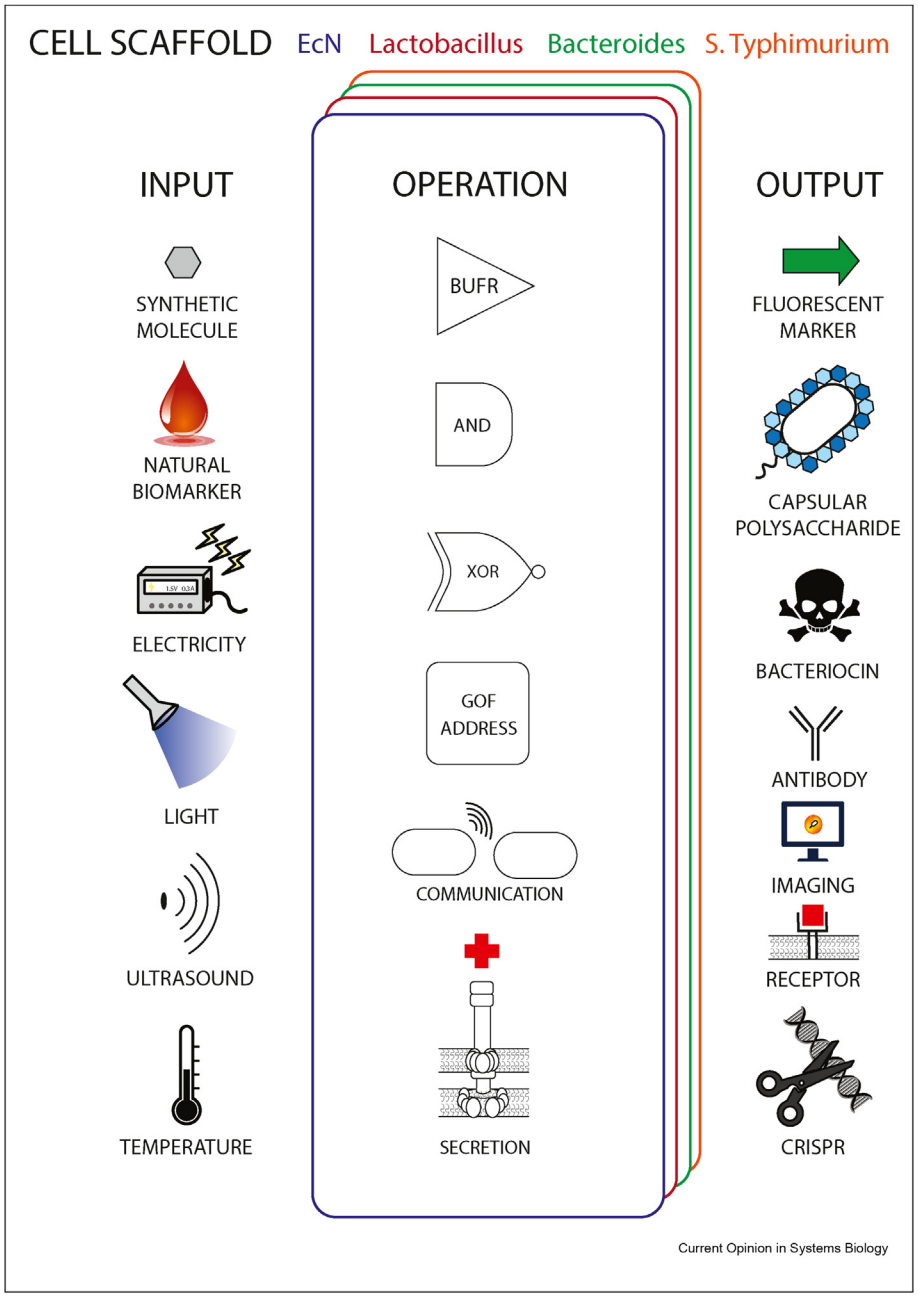
There is an increasing trend in building more complex logical operations to digitally process multiple inputs [8]. Recently, Huang et al. demonstrated all two-input logic gates in five strains of *Bacteroides* using transcriptional programming [4]. This study also improved the concept of circuit compression [9] by requiring fewer transcriptional regulators and regulated promoters, thereby significantly reducing metabolic burden upon the cell. This approach establishes a new paradigm for designing advanced logical computing in a human gut-relevant microbial strain.

Table 1

Summary of engineered living therapeutics.

Input	Operation	Output	Cell scaffold	References
Section 1: Regulatory operations				
Human GI biomarkers	BUFFER operation	Fluorescence marker	*EcN*	[6]**
Synthetic chemical (IPTG)	BUFFER operation	Capsular polysaccharides	*EcN*	[7]
Microbial autoinducers	BUFFER operation in COMMUNICATION	Bacteriocin to kill *S. aureus*	*E. coli*	[12]
Synthetic chemical (IPTG)	BUFFER, AND, XOR	Fluorescence marker	*Bacteroides supp.*	[4]**
Natural sugar (D-ribose)	BUFFER, AND, XOR	Fluorescence marker	*Bacteroides supp.*	[4]**
Human biomarker (nitrous oxide)	BUFFER (NorR) and MEMORY (FimE)	Therapeutic payload	*S. typhimurium*	[10]
Natural sugar (L-arabinose)	BUFFER operation	TNFα antibodies	*E. coli*	[16]
Natural sugar (L-arabinose)	BUFFER operation	Engineered type 3 secretion system	*S. typhimurium*	[14]
Section 2: Physical biosensing modalities				
Ultrasound	Temperature-sensitive BUFFER and MEMORY	αCTLA-4 or αPD-L1 nanobodies	*EcN*	[18]
Ultrasound	Acoustic-sensitive BUFFER operation	Ultrasound imaging	*EcN*	[19]**
Voltage	Redox-sensitive COMMUNICATION	Therapeutic payload	*E. coli*	[24]**
Light	AND operation	IL-10	*EcN*	[22]
Section 3: Microbial cell scaffolds				
Natural peptide pheromone	BUFFER operation	Recombinant AgE6 antigen	*L. plantarum, L. brevis, L. curvatus, L. rhamnosus, L. sakei, L. gasseri, L. acidophilus, L. reuteri*	[34]
Natural sugar (L-arabinose)	BUFFER operation	Therapeutic payload	*EcN*	[38]
Human biomarker (deoxycholic acid) and synthetic inducer (aTC)	XOR operation	Luciferase	*Bacteroides*	[37]
Synthetic inducer (aTC)	BUFFER	CRISPR/Cas system	*Bacteroides*	[39]*

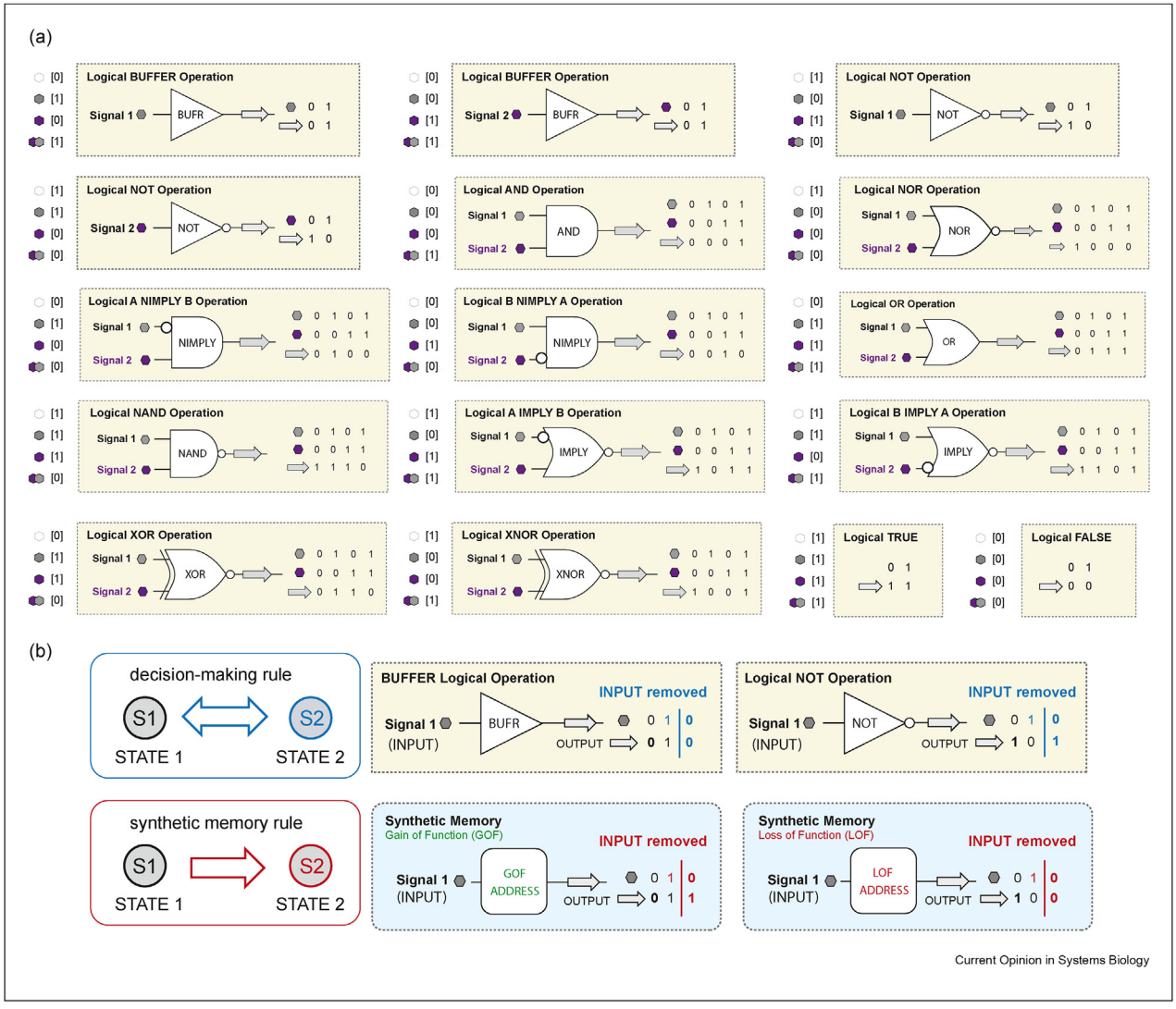
Figure 1



Summary of inputs, operations, outputs, and scaffolds covered in this review. The inputs consist of synthetic molecules, natural biomarkers, electricity, light, ultrasound, and temperature. The operations are the logical buffer (BUFR), AND, and XOR gates, memory-based gain of function (GOF), intercellular communication, and programmed protein secretion. The outputs are fluorescent markers, capsular polysaccharides, bacteriocins, antibodies, imaging, therapeutic receptors, and CRISPR. Therapeutic cell scaffolds are *E. coli* Nissle 1917 (EcN), *Lactobacillus* supp., *Bacteroides* supp., and *S.* Typhimurium. These distinct components can be assessed modularly in preparation of an engineered living therapeutic.

Genetic memory operations can build upon transient decision-making mechanisms for permanent, multi-generation control. Recently Qin et al. engineered a genetic switch in *Salmonella* Typhimurium for tumor detection [10]. They leveraged the increase in nitric oxide levels when *S.* Typhimurium colonizes a tumor and tuned the NO-sensitive regulatory NorR protein to produce the recombinase FimE. FimE subsequently recombines the inverted promoter of a fluorescence marker. This gain of function (GOF) memory address will remain in its new state irrespective of the presence of the input inducer (Figure 2b). The next-generation of synthetic biology memory promises faster recombination speeds (writing) complemented by greater

Figure 2



Summary of (**a**) all 16 Boolean logical operations and (**b**) reversible decision-making and irreversible synthetic memory design rules. (**a**) Full set of 16 two-input logic gates. To the left of each box are the inducer combinations followed by the output state in brackets. Each box depicts the logical gate with the corresponding truth table to the right. (**b**) Logical decision-making processes are reversible between two states. Once the input inducer has been removed, the circuit reverts to its starting state. In contrast, synthetic memory is a permanent genetic modification whereby an input is mapped to either a gain of function (GOF) or loss of function (LOF) address. Both addresses maintain the output state even after the original input is removed.

programming capability, in addition to reduced metabolic burden on the cell scaffold [11]. This capability will enable advances in combinational living therapeutic treatments and diagnostic modalities in the future.

Modern research expands intracellular mechanisms to coordinate function on a multicellular level. Cell to cell communication is typically facilitated by quorum sensing, whereby individual microbes integrate population-based cues to express a large range of phenotypes. This principle can be extended to microbes of different species, unlocking a new paradigm for ELTs. Benítez-Chao et al. demonstrated how *E. coli* can be engineered with the AgrC/AgrA regulatory operon to detect methicillin-resistant *Staphylococcus aureus* (MRSA) autoinducer peptides [12]. As the autoinducers are a molecular proxy for cell growth, at high cell densities *E. coli* is alerted to MRSA's increased presence and produces a positively regulated bacteriocin. This secreted toxin selectively kills MRSA without the need for an external inducer. These autonomous bacterial sentinels can defend the human body from unwanted pathogenic invaders.

Beyond transcriptional frameworks, an ELT also requires rational design of downstream regulatory operations. To improve spatial precision in microbial therapeutics Lynch et al. designed strains of EcN, known as

PROT$_3$EcT, with a programmable protein secretion system [13]. Three modular components-a protein secretion system, transcriptional activator, and therapeutic payload-are used to secrete either nanobodies or tumor necrosis factor-alpha (TNF- α) neutralizing nanobodies. The authors discovered that their technology was equivalent to systematically administered anti-TNF-α monoclonal antibodies in suppressing the development of colitis. Chabloz et al. used a similar approach by enhancing the type III secretion system of *S.* Typhimurium for protein delivery into the cytosol of eukaryotic cells [14].

The diversity of regulatory operations affords scientists programmable genetic frameworks to deploy as desired. Future solutions will need to address practical challenges associated with increasing the complexity of input signals or operations. These include a larger metabolic burden and the need for more precise control.

Physical biosensing modalities

The prevailing approach in ELTs involves the use of chemical inducers as inputs [7] or the implementation of sense-and-response systems [15] to activate engineered microbial solutions. These strategies can more easily facilitate the transition from *in vitro* experiments to *in vivo* therapeutic applications. Their robustness, minimal requirements for administration, and mechanistic familiarity have all contributed to the development of a wide range of circuit designs. Despite the advantages of these traditional methods, non-biochemical exogenous inputs have begun to gain more attention in recent years [16,17]. These physical biosensing modalities provide wide applicability in various interdisciplinary therapeutic contexts.

In this regard, focused ultrasound (FUS) activated genetic circuits within engineered EcN have been effective as a localized immunotherapy approach for cancer treatment. FUS provides deep tissue penetration with radiofrequency waves to subsequently activate a designed therapeutic agent through heating. Abedi et al. recently combined FUS with a temperature-sensitive transcription factor, which permitted recombinase activity to switch state and secrete αCTLA-4 or αPD-L1 to target T-cells [18]. More recently, researchers have targeted gas vesicles using FUS for non-invasive tumor imaging [19]. This innovative approach combines regulatory operations with a distinct physical biosensing modality to offer deeper *in vivo* resolution of the microscale interactions.

Similarly, light has surfaced as a highly effective trigger in prokaryotic research, showcasing superior and unique abilities in controlling gene expression [20]. Optogenetics is a technique that harnesses light to regulate biomolecular activity and offers numerous advantages, including precise spatiotemporal control, exceptional reversibility, straightforward manipulation, and minimal disruption of native biomolecules [21]. However, a major challenge lies in devising methods to effectively deliver light to host organisms where the bacteria are deployed for therapeutic applications. The solution to overcome such limitations can be discovered through interdisciplinary studies involving biology, nanotechnology, or materials engineering. Upconversion microspheres (UCM) possess the ability to absorb near-infrared (NIR) light and subsequently emit blue light. UCM were deployed in the gut with genetically engineered bacteria specifically designed to secrete IL-10, a therapy for ulcerative colitis [22]. As NIR can penetrate more deeply into the skin, external illumination lets the microspheres emit blue light. This subsequently activates pDawn, which is a pre-defined light-responsive two-component system, and ultimately expresses and releases the therapeutic cargo [23].

Electricity is another physical biosensing modality that can be interfaced with biological systems. Terrell et al. have demonstrated a method for integrating electronic devices with engineered microbes through controlled, bidirectional biochemical redox processes [24]. Advanced programmable microbial communities have been coordinated with this local biological network (BioLAN). Future developments in this field will facilitate embedded cross-platform technology across wearable interfaces, living materials, and ingestible sensors [25–28].

Microbial cell scaffolds

Integrating ELTs *in vivo* involves careful consideration of the microbial cell scaffold. Selection of a particular scaffold depends on its natural availability, colonization ability, immunoregulatory properties, genetic manipulability, protein secretion capabilities, and genetic toolbox. In addition to these individual considerations, interactions with the larger microbial population become important. The human body contains numerous micro-organisms collectively known as the microbiome [29]. Various organs like the skin, vagina and gut have distinct microbiomes that uniquely affect human health. With regards to ELTs, recent studies have targeted health disparities in the vaginal or gut microbiomes.

Vaginal health is vital to human reproduction and public health [30]. The vaginal microbiome (VMB) consists of a large microecosystem that produces antimicrobial and anti-inflammatory factors [30] to sustain vaginal health. Disruptions to the VMB can lead to preterm labor, bacterial vaginosis (BV), and increased susceptibility towards sexually transmitted diseases [31]. In a healthy state the VMB is dominated by members of the *Lactobacillus* genus, making them a prime target for ELTs. Generally, these micro-organisms are Gram-positive

aerotolerant anaerobes that have high colonization capacities. *Lactobacilli* supports the VMB by producing lactic acid towards antimicrobial activity, and some species are generally recognized as safe (GRAS) to facilitate safe payload secretion [32,33]. The natural inhabitance and lactic acid production of *Lactobacilli* can be utilized to restore a heathy-state VMB by optimizing its proliferation and lactic acid production capacity to maintain the vagina's acidic pH and outcompete other pathogenic microorganisms. Recently, multiple members of the *Lactobacillus* genus were studied for the production and surface-display of a recombinant mycobacterial antigen for the treatment of tuberculosis both *in vitro* and *in vivo*. Traditional genetic cloning techniques were used to integrate the AgE6 fusion protein, containing the Ag85B and ESAT-6 antigens of *M. tuberculosis* [34]. This engineered *Lactobacillus* contributes to the expanding engineering toolbox for *Lactobacilli* that can be geared towards the motivation of manipulating the VMB with microbial living therapeutics.

While the vaginal microbiome is more localized, the gut microbiota plays a key role in regulating the body's health including immune regulation, metabolism, brain functions, and food digestion [35,36]. Its centrality to overall health makes it an optimal target for ELTs. EcN and *Bacteroides* are model cell scaffolds to modulate the gut microbiome [37,38].

EcN is a non-pathogenic, Gram-negative facultative anaerobe with latent anti-bacterial, and anti-inflammatory activities [39—41]. EcN improves the intestinal barrier function to defend against pathogenic organisms, and to thrive in an inflamed gastrointestinal (GI) tract [38,42]. It also is a readily programmable microbe due to its genetic similarity to wild type *E. coli* [43]. Arabinose induced genetic circuits within engineered EcN have been effective in promotion of the colonic mucosal barrier healing in murine models with dextran sodium sulfate (DSS) induced inflammation. Upon induction, a fusion of the self-assembling protein CsgA with trefoil factors (TFFs) was extended from the surface of the bacteria to promote mucosal healing [38]. Although EcN is a well-characterized gut commensal microbe, its transient colonization of 7—10 days [44] motivates the need for cell scaffolds with long-term colonization when dealing with prolonged disease [38,42].

In this regard, *Bacteroides* have become an attractive scaffold due to their prevalence and ability to efficiently colonize the human gut [4,39,45,46]. *Bacteroides* are Gram-negative obligate anaerobes that compose approximately 25% of colonic bacteria [37,47]. As gut commensals, they naturally maintain long-term colonization to promote local microbiome health. They also metabolize complex molecules as an energy source for the body and protect against pathogenic invasion [47]. Deoxycholic acid (DCA), a secondary bile acid, and anhydrotetracycline (aTc) induced sensors have been successfully engineered to respond to different target environments. This complex two-input, three-output XOR logic circuit enabled *Bacteroides* to differentiate between fermenter, gut, and the post-treatment environment absent of bile acid and inducer [37]. In another study, a highly efficient and versatile CRISPR/Cas-based system was developed, enabling markerless gene deletion and insertion in the *Bacteroides* species [39]. Similarly, Huang et al. achieved the regulation of endogenous genes through loss-of-function with the coupling of CRISPR interference and transcriptional programming [4]. These studies contribute to the expansion of the genetic tools available for use in ELTs.

Although microbial cell scaffolds hold potential for the *in vivo* integration of ELTs, certain challenges related to their long-term colonization must be considered throughout the engineering process. These challenges encompass potential unintended consequences resulting from the environmental release of genetically modified organisms, the need for long-term stability and maintenance, the potential for host immune responses hindering sustained colonization, and the necessity of maintaining and continuously monitoring the introduced microbial population.

Conclusion

Engineered living therapeutics present a promising new frontier for modern medicine. Recent reviews focus more on the mechanism of action of ELTs towards therapeutic solutions [3,48,49]. In this review we consider the simultaneous influence of regulatory operations, physical biosensing modalities, and chassis cells when designing any ELT. This *a la carte* 'menu' of microbial tools can be assembled to create either a therapeutic or diagnostic tool. While many studies have assessed the effects of any one or two of the group, none have yet to iterate amongst all three. This holistic approach can help address some of the limitations of ELTs-including protecting designed solutions against evolving antimicrobial resistances [50,51], constructing orthogonal responses to a diversity of inputs [4,52,53], directing therapeutic localization [54,55], among other considerations. As the synthetic biology toolbox continues to mature, more ELTs can offer solutions for new diagnosis methods and medicines.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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