SPECIAL FEATURE: PERSPECTIVE

Synthetic biology devices for in vitro and in vivo diagnostics

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There is a growing need to enhance our capabilities in medical and environmental diagnostics. Synthetic biologists have begun to focus their biomolecular engineering approaches toward this goal, offering promising results that could lead to the development of new classes of inexpensive, rapidly deployable diagnostics. Many conventional diagnostics rely on antibody-based platforms that, although exquisitely sensitive, are slow and costly to generate and cannot readily confront rapidly emerging pathogens or be applied to orphan diseases. Synthetic biology, with its rational and short design-to-production cycles, has the potential to overcome many of these limitations. Synthetic biology devices, such as engineered gene circuits, bring new capabilities to molecular diagnostics, expanding the molecular detection palette, creating dynamic sensors, and untethering reactions from laboratory equipment. The field is also beginning to move toward in vivo diagnostics, which could provide near real-time surveillance of multiple pathological conditions. Here, we describe current efforts in synthetic biology, focusing on the translation of promising technologies into pragmatic diagnostic tools and platforms.

synthetic biology | diagnostics | biosensing | synthetic gene networks | nanobiotechnology

Synthetic biology employs a forward-engineering approach to create new molecular function. In the field's earliest stages, engineering principles guided the design and construction of synthetic gene regulatory circuits, such as toggle switches and ring oscillators (1, 2), which quickly led to the development of logic, sensor, counter, and timer elements (3). These capabilities continue to grow in complexity and now include biomolecular circuits that can interrogate both intra- and intercellular spaces and, in response, direct downstream activity of other engineered components, as well as endogenous cellular elements. Although there is not a clear consensus regarding a definition of the boundaries of synthetic biology, it is widely accepted that squarely within them is the aspiration to use synthetic circuitry and other engineered components to create novel functions inside cells. In the context of diagnostics, synthetic biology design efforts are typically focused on building sensors that are coupled to a measurable output. These circuits are the outcome of the engineered assembly of natural molecular components, which have been rewarded with survival for their functional performance through eons of natural selection. Biology is enriched with an incredible molecular diversity of sensors and regulators that help maintain organism homeostasis, find resources, and avoid deleterious stresses. Synthetic biologists are beginning to draw on this diversity of sensors and regulatory elements, incorporating them into gene networks and applying their own design criteria of selection for use within novel synthetic architectures. Here, we describe how this rationale is being applied to confront the growing need for novel diagnostic tools and capabilities.

Whole-Cell Biosensing

Hybrid Devices. Synthetic biology was recognized early on as an opportunity to engineer organisms that could serve as whole-cell biosensors in what may be viewed as an extension of the use of animal sentinels for environmental sensing throughout history. Examples of the latter include caged canaries warning of toxic gases in coal mines, the centuries-old practice of using hogs to locate rare truffles, or training dogs to detect illicit materials, explosives, or even cancer (4). In the years preceding the emergence of synthetic biology, animal sentinels were scaled down to the essential unit of life: the living cell. Early cell-based biosensors were a physical merger between single-cell organisms and hardware components that could transduce biochemical signals into a measurable electronic output. Mounted onto electrodes, the cells functioned as the sensor component of the larger detector. In one example, photosynthetic cyanobacteria were used as biocatalysts that reduced an electrochemical mediator, $[Fe(CN)_6]^{3-/4-}$, which then donated an electron to the electrode, producing a steady current flow that correlated to photosynthetic activity (5). This activity and the ensuing current were disrupted by herbicides and, therefore, could report their presence in tested water. In another example, *Zymomonas mobilis* was immobilized on a pH electrode, and its native glucose–fructose metabolic activity was used to measure sugar levels via hydrogen ion production (6).

These native cell-based technologies carried important advantages over abiotic sensors based on purified antibodies or nucleic acid hybridization, including lower cost, improved stability, and the capacity to generate higher biocatalytic activity (7, 8). However, whereas these early biosensing devices were remarkable, they suffered from poor selectivity and interference from culturing conditions (9). As the genetic modification of microorganisms became more practical, synthetic whole-cell biosensors were engineered such that the microorganism encompassed both purpose-built analyte sensors and signal output components. Thus, rather than being

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constrained by naturally evolved systems, the organism itself became the target of engineering efforts (10). Designer circuits tuned for specific molecular recognition, rather than a general effect on host physiology, provided a significant step forward for biomolecular selectivity. In many early embodiments, the signaling element relied on genetically introducing luciferase gene cassettes for bioluminescent reporting. For instance, by fusing the *Vibrio fisheri luxCDABE* cassette to a naphthalene degradation operon in *Pseudomonas fluorescens*, these living biosensors could sense naphthalene and salicylate bioavailability and produce a bioluminescent response (11). Over time, bacterial strains were genetically modified to detect arsenic in natural water resources (12), as well as families of other analytes, including hydrocarbon pollutants, sugars, heavy metals, and antibiotics (13).

The integration of living cells with machines was a trend that persisted through the early days of synthetic biology, with the coupling of genetically engineered whole-cell sensors to electrochemical, optical, or mechanical transducers. Compact and portable hybrid devices incorporating whole-cell sensors directly mounted onto integrated circuits enabled bioluminescent quantification outside of the laboratory (9, 14). Other examples include devices that mimicked natural biofilms by using protective cellular encapsulation in polymers, or whole-cell biosensing arrays for multiplexing (9, 15, 16). The trend of hybrid devices has mostly been abandoned for the convenience of plate readers because data can be collected using this platform in an automated and high-throughput fashion. Interestingly, this convenience may have had the unintended consequence of drawing synthetic biology toward laboratory applications rather than deployable tools. However, as efforts move toward pragmatic applications, especially those that may be applied in the field, the revitalized integration of companion electronics with engineered cells may be an important path forward.

RNA-Based Biosensing in Living Cells. As synthetic biology has matured, increasing gene circuit complexity has allowed for greater sensitivity and reporter tunability (17). We now have the potential to expand biosensing to metabolites and disease-specific RNAs, including sequences imparting drug resistance. Like proteins, RNA molecules can serve as biological catalysts and genetic regulatory elements. Examples of the latter occur naturally (18–20) and have, more recently, become the target of engineering efforts to harness RNA-based sensing through riboregulators and Spinach RNA for small molecules and target RNA sequences (21–25). RNA sensing for diagnostics recently took a leap forward with the development of a new class of RNA sensors called toehold switches (26). Until this point, riboregulators relied on the sequestration of the ribosomal binding site (RBS), via a cis-repression sequence, to control the translation of the downstream mRNA (Fig. 1*A*). In the presence of a higher affinity RNA molecule (the transactivator), the RBS is exposed and translation ensues. Although useful, riboregulators are limited for diagnostic applications because the transactivator must share sequence conservation with the RBS, which is not a distinguishing feature of real-world mRNA sequences. Green et al. (26) resolved this constraint by adding a "toehold" for trigger RNA binding and positioning the RBS in a loop region of an RNA hairpin, which prevents ribosomal binding and reporter translation while eliminating the need for sequence conservation between the transactivating component and the RBS (Fig. 1*B*). Translation begins only when the trigger RNA binds the toehold, which unzips the hairpin into a linear state. This approach was further extended to the detection of full-length mRNA in living cells, highlighting the potential toward intracellular RNA diagnostics. Importantly, toehold-based sensors can be rationally designed de novo by placing the complement of the target sequence in the 5′ region of the toehold. Using these toehold sensors, bacteria were programmed to detect both short synthetic RNAs and full-length endogenous mRNAs within the cellular environment.

Such RNA-sensing technology has great diagnostic potential; however, while confined within the structure of the bacterial cell, its accessibility to target RNA in clinical samples is limited and, therein, its range of application, as well. This example and whole-cell biosensors in general are also inherently bound to biosafety concerns with regard to the release of genetically modified microorganisms into certain environments. Therefore, one of the challenges and opportunities that synthetic biology faces in the development of deployable diagnostics is to circumvent such limitations when possible. Consequently, focus has recently been trained on practical approaches for hosting these powerful tools outside of the cell. As we discuss below, there are new technologies that have successfully brought the rational design of synthetic biology to in vitro diagnostics.

In Vitro Diagnostics

Phage-Based Diagnostics. Although conventional antibody-based diagnostics have, in many ways, met the growing need for in vitro diagnostic tools for clinical and global health, the rational design principles of synthetic biology promise a revolution toward the development of new diagnostic tests for a

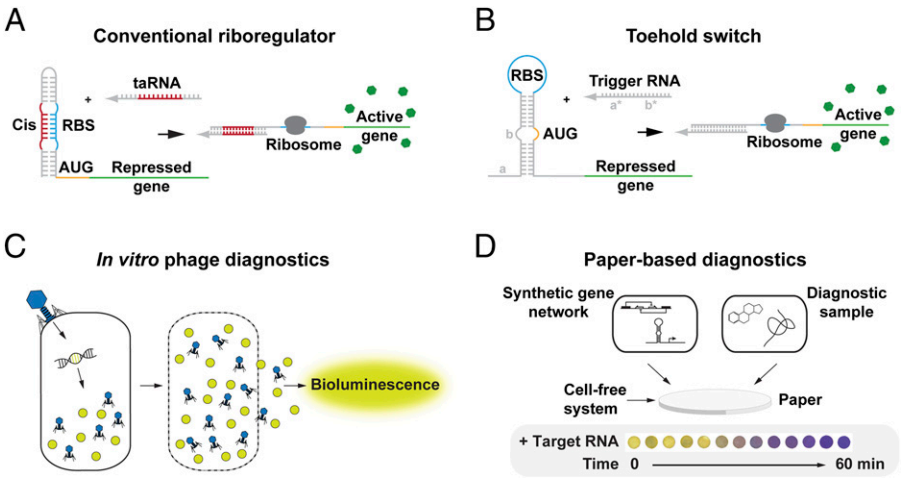

Fig. 1. RNA-based biosensors and synthetic biology platforms for in vitro diagnostics. (*A*) Conventional riboregulators inhibit the translation of mRNA from the start codon (AUG) by sequestering the RBS through a cis-repression sequence (Cis), which is relieved in the presence of the transactivator RNA (taRNA). (*B*) In the toehold switch model, the RBS is located in a hairpin loop within the repressed RNA's 5′ untranslated region and a toehold is added to the 5′ end. This alternative regulatory RNA structure allows for a much larger RNA sequence space to be detected. (*C*) In vitro phage-based diagnostics rely on specific recognition of target bacterial species by engineered phage particles. Once the phage has bound, the engineered phage genome is injected into the targeted cells, where the reporter gene is expressed (i.e., luciferase; yellow circles) and phage replicate. (*D*) Paper-based systems are assembled by freeze-drying a diagnostic gene network and a cell-free coupled transcription/translation system into paper or other porous materials. The gene circuit becomes active when rehydrated with the test sample, containing target RNAs or small molecules.

fraction of the cost and time. Engineered bacteriophage are leading the advance into this space. These bacterial viruses harbor natural specificity to a wide range of bacterial pathogens and can be used to transform target bacteria into factories for reporter molecules. In diagnostic applications, the presence of the correct bacterial surface epitope serves as a conditional input that regulates the transfer of the engineered phage genome into the bacteria, leading to luciferase or other reporter gene expression (Fig. 1*C*). Phage binding specificity can be further optimized through directed evolution, allowing for highly selective reporting even within complex bacterial cultures, prompting many groups to engineer synthetic phage platforms that can identify within minutes contamination by specific bacterial strains (27, 28). Phage also have the advantage of being inexpensive to manufacture because the material for 10^7 different tests could theoretically be generated from a 100-L fermenter (29). Using such techniques, there are now commercially approved products for microbial detection in industrial settings, and clinical applications have been demonstrated as well (28). One compelling example is based on an engineered variant of the CDC plague diagnostic phage, ϕA1122, for *Yersinia pestis*, the etiologic agent of the bubonic plague (29, 30). The bacteria can be detected through expression of a phage-vectored bioluminescence reporter within 40 min in liquid culture or 120 min in human serum. Other clinical demonstrations include reporter systems for *Bacillus anthracis* (31) and *Mycobacterium tuberculosis* identification. These methods can be combined with drug treatment to gather drug susceptibility data along with species identification (29, 32). Due to limited bacterial concentrations in test samples, an enrichment stage is sometimes required that, in the future, could perhaps be remedied by low-cost microfluidics coupled with a pathogen capture technology, such as opsonin-coated beads that recognize the carbohydrate pattern on many clinically relevant bacteria (33). This approach would also allow for the removal of reporter-quenching contaminants, like hemoglobin, and delivery of optimal medium for phage infection (34).

Paper-Based Synthetic Gene Networks. A new concept takes in vitro synthetic biology a step further by completely removing the cellular context and putting synthetic gene circuits on paper (Fig. 1*D*) (35). In this approach, commercially available cell-free transcription/translation systems (bacterial or mammalian) are freeze-dried onto paper or other porous substrates to create poised genetic regulatory networks that are stable for long-term storage at room temperature and are activated by rehydration. This work follows on other efforts in cell-free synthetic biology, which have provided important dynamic and mechanistic insight on gene regulatory networks and allowed for rapid "build-test" cycles for prototyping engineered gene circuits and biomanufacturing pathways (36–41). The in vitro nature of these systems resolves the challenge faced by cell-based approaches of importing biomolecular components into the intracellular space, making these cell-free environments easily modified and excellent platforms for engineering.

The paper-based synthetic biology platform provides a mode for safe and sterile deployment of engineered gene circuits and was demonstrated by incorporating toehold switches and other circuit designs, exhibiting small-molecule and RNA actuation. Importantly, the platform was also used to host completely abiotic gene-based sensors for glucose and mRNAs, including diagnostic assays for four antibiotic resistance genes and 24 Ebola sensors (35). The Ebola sensors could distinguish between Sudan and Zaire strains and were developed in under 12 h for a cost of just over $20 per sensor, a time and cost regime that compares very favorably with antibody-based diagnostics. In addition to fluorescent reporter output, these sensors were designed to produce enzymes that generated color changes visible to the naked eye, a necessary feature for low-resource environments lacking technical infrastructure. Similar to early whole-cell synthetic biology demonstrations, the paper-based reactions were merged with custom, low-cost electronics for quantification and automation of diagnostic reactions in the field.

After these proof-of-concept demonstrations, several challenges remain for the practical implementation of paper-based diagnostics, including meeting the detection thresholds required for field use. To overcome this challenge, amplification strategies for incoming molecular signals (RNA/DNA) could be adopted from other applications (33, 42). Using such platforms, both pathogen identification and the recovery of other clinically relevant data could be achieved, such as surveillance of neuraminidase and hemagglutinin composition in seasonal variants of influenza, allowing for early antiviral intervention. Toehold-based sensors promise to play a prominent role in a variety of diagnostic applications (26), but there are also many other technologies and approaches in development that could be paired with the paper-based platform, including directed evolution schemes, prospecting for naturally occurring transcription factors, and the rational design of small-molecule sensors for the detection of metabolites, hormones, or drugs in patient samples (13, 43–45). Also, a recently developed protocol has streamlined the production of cell-free systems, enabling synthetic biologists to generate tailored biochemistry (46). An intriguing example could be to develop systems in which the gene circuit product is degraded in a targeted manner, sparing the translational machinery and allowing repetitive analyte tracking (47, 48). Incorporating transcription-only gene circuits would result in simpler and faster systems. These systems could include using fluorescent Spinach RNA as a direct gene circuit reporter, Spinach RNA sensors that activate in the presence of their programmed target molecule, and Spinach.ST RNA for the detection of DNA and RNA sequences (21, 22, 24).

In Vivo Diagnostics

As detailed above, one of the aims of synthetic biology has been the engineering of organisms to sense the environment. This goal is now being extended to the mammalian in vivo environment and also the engineering of mammalian cells. As the expertise of modeling and implementing synthetic circuits has grown, so has the practicality of using engineered cells (both microbial and mammalian cells) as in vivo biosensors. Circuits with simple logic operations have been expanded into networks with complex integrative logic and memory, which can be used for this purpose. In the near term, in vivo diagnostics hold great promise as research tools to gain better understanding of disease and host-response in situ and, going forward, could be a powerful means for near real-time monitoring of a wide range of medical conditions. It is important to note that, although there is a difference in the nature of biosafety concerns related to the use of genetically modified whole-cell biosensors in the environment, there are also clear safety concerns that need to be meticulously examined for the use of engineered cells for in vivo diagnostics and therapeutics.

Bacterial Cell Hosts. In early efforts to develop bio-based counting and memory storage, synthetic circuits were devised in *Escherichia coli* (*E. coli*) using riboregulator or recombinase designs (30). In the first of these architectures, sequential inducer pulses were counted using riboregulator-mediated translational repression of T7 polymerase (T7 RNAP) and a reporter transcript. In the latter, recombinase cascades were strung together, with each pulse "flipping"

an invertase unit, leading to the expression of the next recombinase in line and ending with reporter gene expression. These models gave rise to the notion that in vivo environmental cues could drive memory units, transforming bacterial host cells into diagnostic indicators for recording exposure events. One possible use could be the nondestructive interrogation of the human gut microbiome, which is intertwined with health, metabolism, and a growing list of disease states or conditions (49). To this end, *E. coli* were programmed to serve as "living diagnostics" and report environmental signals in the mouse gut (Fig. 2*E*) (50). This concept was accomplished by implanting the bacteria with a memory element based on the phage lambda *cI/cro* genetic switch (51). A companion trigger element could detect anhydrotetracycline (ATc) (dosed into the mice's drinking water) and switch a *lacZ* reporting unit within the memory circuit to a stable "on" state, recording the event for retrieval from fecal samples and interpretation. Thinking to the future, sentinel bacteria could reside in the guts of soldiers or aid workers and serve as short-term "medical records," alerting on the time and scale of contamination or pathogen infection. Such in vivo monitoring could also be useful for tracking gut health status in patients with inflammatory bowel disease, detecting presymptomatic changes, and reducing the need for more invasive tests, such as endoscopy. Future approaches could combine diagnostic strains for multiplexed sensing, perhaps through the conditional production of antibody-labeled phage that are tracked with a companion device based on an inverse ELISA, where phage-displayed antibodies would be detected using antigen-functionalized gold nanoparticles in a lateral flow test.

Much like bacteriophage can be used for their natural homing capabilities, bacteria can be used to target their preferred in vivo niches. Microorganisms that migrate to and preferentially accumulate in tumors, due to their low-oxygen content, local immune suppression, irregular blood supply, and nutrient availability, can be synthetically modified to assess and report tumor status (52). In one study, nonpathogenic commensal bacteria were genetically modified to express the *luxCDABE* operon, endowing them with bioluminescent qualities (Fig. 2*B*) (53). After injection into mice carrying s.c. xenograft tumors, these strains, in conjunction with bioluminescent 3D optical tomography combined with micro-CT scanning, were used to visualize the malignant growths. To reach deeper tissues in patients and even multiplex output, next-generation systems could be extended to the expression of protein-based contrast agents (45, 54–56). Using heme-binding proteins and other reporters from functional magnetic resonance imaging, future applications could possibly report from deeper tissue, using orthologous reporters for greater information retrieval.

In a recent study, probiotic bacteria (termed, PROP-Z) engineered to express both *lacZ* and the *luxCDABE* operon, were used to target, visualize, and diagnose liver metastasis (57). Importantly, the stability of the synthetic circuitry within PROP-Z was secured by using a dual-maintenance strategy that included the expression of the *Bacillus subtilis alp7* gene (leading to filament production that ensures equal plasmid segregation upon cell division) (58), as well as a toxin–antitoxin system that forces the cell to either maintain the plasmid or die (59). Oral or i.v. delivery yielded preferential colonization of liver or s.c. tumors, respectively, allowing for bioluminescent imaging. Furthermore, administration of a luciferin/galactose conjugate led to its cleavage by β-gal and the resulting release of luciferin into circulation and subsequent clearance by the kidney, allowing for detection and quantification in urine samples.

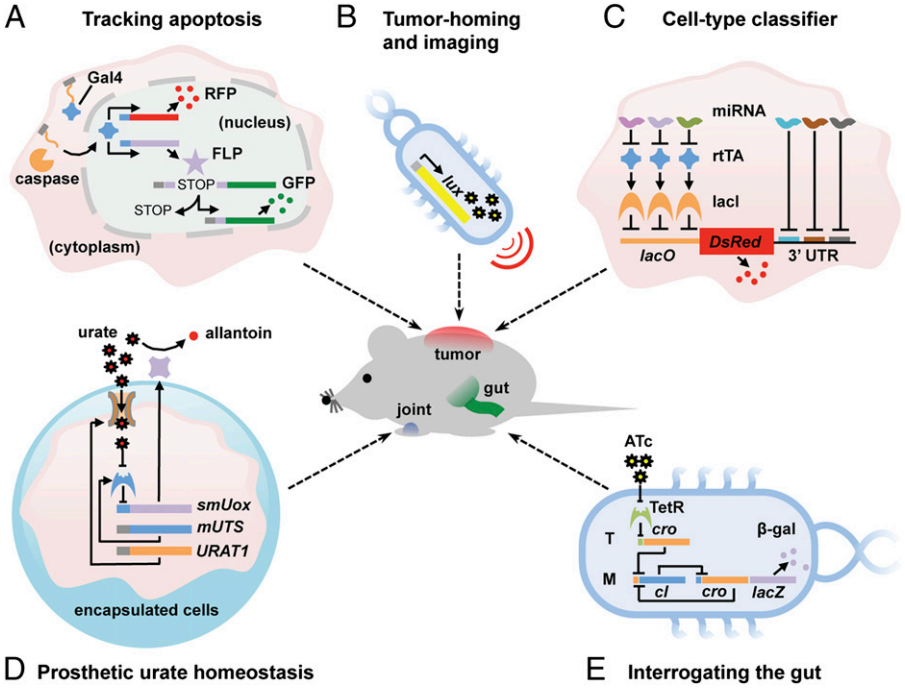

Fig. 2. Synthetic biology devices for in vivo diagnostics. (*A*) The CaspaseTracker: Cytoplasmic Gal4 (blue) is released to the nucleus by apoptotic caspases (orange), where it activates RFP and FLP recombinase (violet star) expression, leading to persistent GFP expression and enabling apoptosis/anastasis tracking (demonstrated in fruit flies). (*B*) Engineered bacteria naturally home in on tumors and express their synthetic circuits, producing bioluminescence for in situ tumor imaging. (*C*) The cell-type "classifier" compares endogenous expression levels of three "high" miRNAs (left) and three "low" miRNAs (right) to a preset HeLa profile. If all of the high miRNAs are above the threshold, this profile leads to RNAi silencing of the transactivator (rtTA; blue) of the output gene's repressor (LacI; orange), allowing for output expression (DsRed; red dots). If all of the low miRNAs are below the threshold, the output mRNA is not degraded via RNAi (demonstrated in cell culture). (*D*) High urate levels lead to tumor lysis syndrome and gout. A prosthetic urate homeostasis system transports urate (red cogs) into the encapsulated cells via constitutive URAT1 (orange) expression, where it releases mUTS's (blue) repression of smUox (violet), a secreted uricase that converts the urate to renally secretable allantoin (red dots). (*E*) Bacteria engineered to record mammalian gut microbiome exposure events. The memory element (M), comprised of a bacteriophage lambda *cI/cro* (blue/orange) switch and containing a *lacZ* reporter (violet), is toggled to the "on" (*cro, lacZ*) state when the Trigger element (T) detects ATc (black cogs) and TetR (green) repression of a second *cro* copy is released. The detection of ATc results in the expression of β-gal from the *lacZ* gene, which is readily measured in fecal samples.

Mammalian Cell Hosts. The preceding examples depict efforts in modifying prokaryotic cells for in vivo biosensing. However, there is also vast potential in using mammalian cells for hosting synthetic gene networks to monitor and/or influence the in vivo environment. For example, researchers have described an alternative to the aforementioned bacteria-based *luxCDABE* bioluminescent imaging system by introducing (and optimizing) the full *lux* cassette into mammalian host cells and successfully demonstrating in vivo imaging using a mouse model (60). Along with a crucial advantage over

platforms that use firefly luciferase or marine aequorin-like proteins—there is no need for exogenous substrate injection before imaging because the expression cassette produces all of the necessary components for bioluminescence (61, 62)—this concept does not entail infection with a bacterial host carrying the synthetic circuit while providing the means for real-time monitoring in live animals.

Engineered gene networks hosted in mammalian cells could also serve as insulated circuits that respond to exogenous input signals or "prosthetic" networks that autonomously sense the intra- or intercellular state, enhancing endogenous pathways or endowing cells with novel theranostic attributes (63). In pioneering work that highlights this potential, a prosthetic network comprised of human, bacterial, and fungal parts was designed for the control of urate homeostasis (Fig. 2*D*) (64). The engineered cells were encapsulated in mice for the treatment of gout and tumor lysis syndrome, which results from the formation of urate crystals in the joints and kidneys, respectively. This system was able to restore urate homeostasis through detection of high urate levels and concomitant derepression of a fungal urate oxidase, a urate-metabolizing enzyme that has been lost in humans (65, 66). Efforts continue to be directed toward engineering dynamic mammalian circuits, including schemes that combine drug-based and gene-based approaches. In one such work, mammalian cells were implanted with a synthetic signal cascade that, when stimulated by an antihypertensive drug (guanabenz), prompted two metabolically active peptides (GLP-1 and leptin) to be secreted, in a multipronged strategy to combat the metabolic syndrome (67).

Small regulatory RNAs offer an additional mode of interaction between gene circuits and host gene expression and physiology. Just as metabolite levels can serve as indicators of underlying health deficiencies and be exploited to trigger corrective action, expression profiles can be used to drive synthetic theranostic pathways. This concept governs the cell-type "classifier"—an artificial network that integrates sensory input from microRNAs (miRNAs), discriminating between HeLa and non-HeLa cells (Fig. 2*C*) (68). This network compares the expression levels of six miRNAs to a preset HeLa profile and computes whether to allow the expression of the output gene. The classifier serves as an in vivo diagnostic or therapeutic, regulating the expression of a fluorescent reporter or proapoptosis gene, respectively. RNA profiling networks that categorize biological state could prove invaluable for early disease diagnosis and seem to be especially well-suited for discriminating diseased cells from self in cases of cancer, viral infections, and autoimmune diseases.

Genetically encoded diagnostics can often be repurposed to become therapeutics with a simple molecular cloning step. This ability to change the nature of the output is in stark contrast to drug-based methods. With regard to circuit-based treatments, a commonly desired physiological response is gene-directed cell death via the expression of cytotoxins, siRNA, prodrug-associated enzymes, or natural apoptosis-linked genes. However, various cancer cell lines in which apoptosis has been induced may recover even after passing critical checkpoints such as mitochondrial fragmentation and nuclear condensation, if the therapeutic agent is removed (69). Although caspase-related apoptosis biosensors for fluorescent imaging or immunodetection have been explored, they lack the memory capabilities required to report the recovery from an apoptotic state (70). To diagnose and record apoptosis and anastasis (its reversal), an in vivo CaspaseTracker biosensor system was developed, whose function begins with the apoptosis-induced, caspase-mediated cleavage of a cytoplasmic Gal4 transactivator, followed by its translocation into the nucleus (Fig. 2*A*) (71). There, it activates the immediate expression of red fluorescent protein (RFP) and also that of a flippase (FLP) recombinase, for memory, leading to the permanent expression of GFP that persists throughout the life of the apoptotic/anastatic cell's progeny. This network, developed in *Drosophila melanogaster,* could be applied to a mammalian research platform as a tool to study apoptosis evasion tactics in the context of cancer or even viral infection.

The majority of synthetic gene circuits are based on networks regulated by transcription factors (TFs); the first-generation circuits were largely implemented with natural TFs (e.g., TetR, GAL4, or LacI) and their binding sequences (72). This limited repertoire has been significantly expanded in recent years, with the discovery and decoding of programmable DNA-binding protein factors such as artificial zinc fingers (ZFs), transcription activator-like effectors, and the clustered regularly interspaced short palindromic repeats/Cas system (73). Today, one can target specific sequences in natural and synthetic networks and deliver activities ranging from transcriptional activation/repression to gene editing depending on the functional component fused to the DNA sensing domain (e.g., KRAB, VP64, or Fok I endonuclease) (74). These important developments have created a powerful driving force toward genome editing and the wiring of synthetic circuits that help elucidate the function of their natural counterparts (75, 76).

Despite these advances, rationally designed DNA-binding proteins have not reached their potential regarding sensing capabilities. For example, in most current applications, designer DNA-binding proteins act upon the DNA target itself (e.g., regulation/editing). However, their programmability could also be wielded to recognize pathogenic or oncogenic sequences and drive an amplified reporter signal or a transcriptional response originating from a separate endogenous or synthetic locus. For example, although antiretroviral therapy can suppress viral loads in patients infected with HIV-1, provirus integrated into the genomes of quiescent $CD4^+$ T cells can reemerge upon cessation of treatment (77). DNA-sensing proteins could be designed to detect and report the presence and location of such latent viral reservoirs. ZF pairs fused to split β-lactamase have been applied for in vitro sequence identification (78), and this general concept could perhaps be expanded upon for the in vivo environment. Linking sequence detection to a customizable transcriptional output might provide a way to tailor a response for a desired physiological outcome, such as immune system recruitment or the activation of a kill switch that induces apoptosis. This approach could yield a powerful platform for identifying and combating pathogen infection or oncogenic transformation in vivo, and for endowing mammalian cells with sentinel capabilities for use in a bioreactor setting.

Delivery Challenges. The targeted delivery of genetically encoded tools is a challenge that surpasses that of drug delivery because the cargo needs to reach its destination and undergo expression. One of the earliest and still major strategies used for delivery involves virus-based vectors, including retroviruses, adenoviruses, and adeno-associated viruses, which have been modified to carry engineered payloads and cell-targeting elements (79, 80). Self-assembled virus-like particles (VLPs) and virosomes (virion-like phospholipid bilayer vesicles with surface glycoproteins) can also carry biomolecular cargo (81, 82). Such delivery modes are quite promising, and some have been approved for use in humans. However, biosafety concerns related to human viruses persist and extend to VLPs and virosomes, in which immunogenicity may yet be an issue.

Some methods circumvent the need for targeted delivery and rather rely on the injection and diffusion of harmless biomarkers for point-of-care diagnosis. A recently published tool for the diagnosis of thrombosis

and colorectal cancer exploits the disease-related presence of specific endogenous proteases, which cleave injected synthetic biomarkers mounted on nanoparticles, freeing the reporter elements that are then read via urinalysis (83). This concept could be applied to limited-resource settings or even adapted to serve as a readout for in vivo diagnostic gene circuits. In other recent work involving point-of-care diagnosis, researchers engineered bacteria to serve as biosensors for the analysis of clinical urine or serum samples to detect disease indicators, such as nitrogen oxides and glucose (84). The "bactosensors," which contained integrase-based synthetic circuitry that allowed for digitization and recording of the pathological markers, were embedded in hydrogel beads and reliably detected glucose content indicative of diabetes in urine samples from multiple prediagnosed patients.

Targeted delivery is nonetheless required in most in vivo theranostic approaches, and nanobiotechnology offers a wealth of opportunities for the delivery of synthetic biology devices. For example, researchers have described DNA-based "nanorobots" that were folded into 3D barrels and outfitted with DNA aptamer-based locks for cell type-specific cargo delivery (85). Only cell type-specific surface antigen "keys" can unlock the barrel, which then spills its contents like an entropic spring, labeling the target cell or influencing its signaling pathways. Although there is great potential for such tools, the in vivo susceptibility of DNA-based nanorobots to degradation must be addressed before use in humans; but, encouragingly, nanorobots have been successfully demonstrated in live insects (86). Solutions to resolve degradation issues have been drawn from the architecture of membrane-encapsulated viruses, with lipid bilayer-coated DNA nanostructures displaying dramatically increased in vivo half-life (87). Nanorobots could perhaps be conjugated to flagellated bacteria and "rafted" to targeted tissues via bacterial self-propulsion, similar to a recent study in which the natural tumor-homing ability of *Salmonella typhimuria* was exploited for the delivery of attached, fluorescently labeled microbeads (88). These "bacteriobots" were injected into tumor-harboring mice and were shown to accumulate in the cancerous tissue.

Biological vesicles derived from microorganisms or human cells have also attracted much attention for in vivo delivery and hold promise for synthetic biology applications (89). One recent study focused on outer membrane vesicles (OMVs) that are naturally produced by Gram-negative bacteria (90). OMVs were engineered to display an antibody targeting a receptor that is overexpressed in many malignant tumors and then loaded with fluorescently labeled kinesin-spindle-protein siRNA. Injection into a mouse tumor model allowed for both growth inhibition and in situ fluorescent imaging of the xenograft. A similar delivery option, exosomes (membrane vesicles secreted by mammalian cells), may be even more advantageous over nonbiological materials because they can be produced from a patient's own cells (lower immunogenicity), and their fusion with target-cell membrane may bypass the endosomal–lysosomal pathway and avoid inflammasome activation (91). Engineered exosomes have been applied in both diagnostic and therapeutic applications, including mouse models for cancer and Alzheimer's disease (92, 93). Looking forward, lipid-based vesicles hold the exciting possibility of becoming bio-manufacturing products. For now, vesicles must be loaded postproduction, but it is exciting to consider future avenues in which cell lines will be engineered for automated preloading of the cargo and diagnostic capabilities.

Conclusions and Future Directions

As synthetic biology joins ranks with other medical and scientific fields to tackle global challenges, such as providing low-cost, rapidly deployable diagnostics, success will continue to be found at the interfaces between disciplines. One example may include the use of technology to augment gene circuits, like the toehold switch, with hardware. As discussed here, the engineering approaches of synthetic biology have the potential to significantly advance the translation of promising technologies into pragmatic tools suitable for real-world applications. The paper-based platform is poised to play a prominent role in in vitro applications because it fits the need for low-cost, practical, and simple diagnostic tools for use outside of the laboratory, and the underlying concept may be merged with other technologies and extended in new and exciting directions. Additionally, existing in vivo sensors will begin to transition from their current research models to patient applications for real-time monitoring and early, personalized diagnosis. It is also intriguing to imagine in vitro synthetic biology embedded into diagnostic wearables for patients, medical personal, military personal or even athletes, allowing for both personal and environmental sensing. Bio-based wearables could also serve as an interface for in vivo diagnostics, alerting patients with preexisting conditions to presymptomatic changes in physiology. Ultimately, it will be the creativity embodied in collaborative synthetic biology that will guide this field into the future.

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