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Acoustic reporter genes for noninvasive imaging of microorganisms in mammalian hosts

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SUPPLEMENTARY NOTES

Supplementary Note 1 – Spectral Unmixing. Gas vesicles with different critical collapse pressures can be distinguished from each other by applying acoustic pulses of gradually increasing amplitude and monitoring the disappearance of backscattered signal[1]. One subset of gas vesicles collapses first, followed by another, and so on. A signal processing paradigm similar to spectral unmixing can then be used to determine the contribution of each population to the total signal[1].

Supplementary Note 2 – Host Burden from ARG1 Expression. Some degree of burden is expected to accompany heterologous protein expression[2,3]. To assess the burden on EcN cells presented by ARG1, we characterized their growth, viability, reporter expression maintenance, and release of microcins. The growth of cultures expressing ARG1 and LUX followed a similar time course after IPTG induction (**Extended Data Fig. 8a**), reaching final OD_{600} values of 5.66 ± 0.76 and 8.16 ± 1.12, respectively – a trend toward somewhat higher density for LUX. The quantity of viable colony forming units per OD was also similar between these samples (**Extended Data Fig. 8b**). Most EcN cells transformed with the ARG1 plasmid retained their ability to express gas vesicles after overnight induction (**Extended Data Fig. 8c**); a fraction of the colonies was non-gas vesicle-producing, suggesting that some shutdown of transgene expression occurs in EcN cells during transformation, recovery or growth. EcN cultures expressing ARG1 and LUX both retained their ability to inhibit the growth of the microcin-sensitive bacterial strain *E. coli* K-12 H5316[4] (**Extended Data Fig. 8d**). Overall, these results suggest that ARG1 expression at levels that make EcN cells brightly visible to ultrasound is reasonably well tolerated, with scope for further optimization to reduce burden and ensure reporter gene retention.

Supplementary Note 3 – ARG expression in *Salmonella typhimurium* and intratumoral imaging. Another emerging application of engineered microbes is as antitumor therapies and diagnostics [5-7]. For example, *Salmonella typhimurium* has been engineered to colonize tumors after intratumoral or systemic injection and secrete proteins causing cancer cell lysis and immune system stimulation [5,6]. Being able to image such therapies in the body (**Extended Data Fig. 9a**) could facilitate their development and clinical translation. To enable this possibility, we adapted the genetic construct encoding ARG1 for expression in attenuated *S. typhimurium* strain ELH1301, which has been used in tumor-homing studies [5,6]. Upon induction with N-(β-ketocaproyl)-L-homoserine lactone (AHL), ARG-expressing *S. typhimurium* cells produced abundant intracellular gas vesicles and were readily observable using ultrasound compared to controls expressing the LUX operon (**Extended Data Fig. 9, b-c**). The level of ultrasound contrast was similar to ARG-expressing *E. coli*, with mean intensities per pixel of 9.5 ± 0.7 and

12.1 ± 2.1, respectively, at a density of 10^9 cells/ml. Following pressure-induced collapse, these cells are indistinguishable from luciferase-expressing controls (**Extended Data Fig. 9b**). As a proof of concept, we tested whether ARG-expressing *S. typhimurium* could be imaged *in vivo* in a murine tumor, where these bacteria can proliferate to densities of 10^{10} cells per gram tissue [6]. Live ARG-expressing cells were injected into OVCAR8 ovarian adenocarcinoma xenografts in nude mice at one third of this concentration and imaged with ultrasound. Contrast was readily apparent in tumors containing the engineered *S. typhimurium*, and disappeared after acoustic collapse (**Extended Data Fig. 9, d-e**). Cells expressing the luciferase operon had no discernable ultrasound contrast (**Extended Data Fig. 9e**). These results demonstrate that ARGs can be employed in more than one bacterial species and that bacteria are detectable inside tumors at concentrations relevant to therapeutic and diagnostic applications.

Supplementary Note 4 – Sensitivity of ultrasound imaging and comparison with other methods. To use ARGs in imaging a broader range of cell types and biological contexts, the sensitivity of ARG detection should be improved beyond the level demonstrated in this initial study. Sensitivity improvements may be obtained in future studies through rational engineering or directed evolution of gas vesicle gene clusters. The enhanced ultrasound contrast produced by buoyancy-enriched cells (**Extended Data Fig. 2**) suggests that more efficient ARG expression could, on its own, more than double detection sensitivity. Combined with advanced nonlinear and ultrafast detection schemes[8-10], such improvements could also facilitate imaging in larger organisms. While optical techniques, such as fluorescent, luminescent and photoacoustic imaging, are also becoming more capable of visualizing cells in mice with improved resolution using advanced contrast agents[11-14], photon scattering and absorption make it challenging to scale them up to larger animals or patients. By contrast, ultrasound is intrinsically scalable to human subjects, making it possible to conceive of future clinical applications for ARGs.

Supplementary Note 5 – Heterologous expression, construct stability and host burden. Although *E. coli* Nissle 1917 and *S. typhimurium* are both widely studied human-colonizing microbes and popular chasses for microbial engineering, it will be useful to adapt ARGs to a broader range of bacterial hosts such as the commensal genera *Bacteroides* and *Lactobaccili* or the pathogens *Clostridium* and *Pseudomonas*, as well as recently developed strains of *E. coli* optimized for stable colonization of the gastrointestinal tract[15,16]. Since both of the cell types used in this study are Gram-negative, expressing ARGs in Gram-positive species may require modifying the ARG cassette; the feasibility of such expression is supported by the natural formation of gas vesicles in certain Gram-positive bacteria, such as *Desulfotomaculum*[17,18].

Furthermore, for studies involving *in vivo* colonization, it will be important to maintain ARG construct stability, and ensure that ARG expression does not disadvantage cells in their ability to grow and compete within their niche. Although ARG-expressing EcN cells were able to grow, remain viable and release microcins (**Extended Data Fig. 8**) , trends in our experimental data suggest some degree of burden due to ARG expression, as expected in heterologous expression systems[2,3]. In future studies, this burden could be mitigated by regulating the overexpression of gas vesicles with negative feedback, triggering ARG expression transiently before imaging using two-step circuits (in which the ARG operon is controlled by a chemically inducible transcription factor whose own expression is connected to the circuit of interest), or stochastically expressing ARGs in a subset of the overall cell population to enable unburdened growth by the remaining cells. Additionally, to enhance the long-term stability of ARG constructs, it may be useful to incorporate them into the bacterial genome, or into plasmids containing engineered stability elements[19-22]. Similar approaches were needed to stabilize the *in vivo* expression of LUX, whose burden resulted in the loss of unstabilized plasmid shortly after *in vivo* administration[6,23]. The fact that gas vesicles are expressed naturally in a wide range of bacterial and archaeal species as part of their normal life cycle[18,24] suggests that regulated expression of these proteins is compatible with healthy cellular function.

PLASMID SEQUENCES FOR ARG CONSTRUCTS

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Supplementary Table 1 – Estimated buoyant force on ARG-expressing cells

Fraction of Cell Occupied by Gas	Buoyant Force (fN)
0%	-4.31
10%	0.43
20%	5.17
50%	19.40

This calculation assumes a wild-type *E. coli* cell density of 1.1 g/mL [1], a cellular volume, *v*, of 4.4 fL [2], and gravitational acceleration, *g*, of 9.8 m s^{-2}. The density of an ARG-expressing cell, $\rho_{cell,}$ is calculated as 1.1 g /mL * (1 – *f*), where *f* is the fraction of the volume occupied by the gas in gas vesicles. Buoyant force, *F*, is then calculated as $\boldsymbol{F} = (\boldsymbol{\rho_{media}} - \boldsymbol{\rho_{cell}})\boldsymbol{gv}$, where ρ_{media} is taken to be 1 g/mL. For comparison, the thrust force of *E. coli* flagellar propulsion is 570 fN [3]**.**

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