LETTER

doi:10.1038/nature25021

Acoustic reporter genes for noninvasive imaging of microorganisms in mammalian hosts

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The mammalian microbiome has many important roles in health and disease[1,2], and genetic engineering is enabling the development of microbial therapeutics and diagnostics[3–7]. A key determinant of the activity of both natural and engineered microorganisms *in vivo* is their location within the host organism[8,9]. However, existing methods for imaging cellular location and function, primarily based on optical reporter genes, have limited deep tissue performance owing to light scattering or require radioactive tracers[10–12]. Here we introduce acoustic reporter genes, which are genetic constructs that allow bacterial gene expression to be visualized *in vivo* using ultrasound, a widely available inexpensive technique with deep tissue penetration and high spatial resolution[13–15]. These constructs are based on gas vesicles, a unique class of gas-filled protein nanostructures that are expressed primarily in water-dwelling photosynthetic organisms as a means to regulate buoyancy[16,17]. Heterologous expression of engineered gene clusters encoding gas vesicles allows *Escherichia coli* and *Salmonella typhimurium* to be imaged noninvasively at volumetric densities below 0.01% with a resolution of less than 100 μm. We demonstrate the imaging of engineered cells *in vivo* in proof-of-concept models of gastrointestinal and tumour localization, and develop acoustically distinct reporters that enable multiplexed imaging of cellular populations. This technology equips microbial cells with a means to be visualized deep inside mammalian hosts, facilitating the study of the mammalian microbiome and the development of diagnostic and therapeutic cellular agents.

Gas vesicles comprise all-protein shells with sizes of approximately 200 nm that enclose hollow interiors, and allow dissolved gases to permeate freely in and out while excluding water[16]. We recently discovered the ability of these proteins to scatter sound waves and thereby produce ultrasound contrast[18]. However, the ability of the multi-gene clusters encoding gas vesicles to serve as reporter genes in heterologous species has not been demonstrated. Gas vesicles are encoded in their native bacterial or archaeal hosts by operons of 8–14 genes, which include the primary structural protein GvpA, the optional external scaffolding protein GvpC, and several secondary proteins that function as essential minor constituents or chaperones[17]. As a starting point for developing acoustic reporter genes (ARGs), we chose a compact *E. coli*-compatible gas vesicle gene cluster from *Bacillus megaterium*[19] (Fig. 1a; top left). Although cells containing this construct were able to produce small, bicone-shaped gas vesicles (Fig. 1b, c; left), its expression did not result in bacteria that were detectable by ultrasound (Fig. 1d; left), most probably because the small gas vesicles produced from this construct have weak acoustic scattering. At the same time, transforming *E. coli* with a gas vesicle gene cluster derived from the cyanobacterium *Anabaena flos-aquae*, the gas vesicles of which are highly echogenic[18,20], did not result in gas vesicle expression. Given the high sequence homology of GvpA between organisms (Extended Data Fig. 1), we hypothesized that a combination of the structural *gvpA* genes from *A. flos-aquae* with the accessory genes *gvpR–gvpU* from *B. megaterium* (Fig. 1a; middle) would result in the formation of gas vesicles with characteristics favourable for ultrasound imaging. Indeed, expression of this engineered gene cluster resulted in *E. coli* containing gas vesicles with substantially larger dimensions compared to the *B. megaterium* operon, and these nanostructures appeared to occupy a greater fraction of intracellular volume (Fig. 1b, c; middle). Notably, these cells produced robust ultrasound contrast compared to green fluorescent protein (GFP) controls (Fig. 1d; middle). Further engineering comprising the addition of a gene encoding the *A. flos-aquae* scaffolding protein GvpC (Fig. 1a; right) resulted in wider and more elongated gas vesicles that more closely resembled those native to *A. flos-aquae*[18] (Fig. 1b, c; right), and generated stronger ultrasound contrast (Fig. 1d; right). We refer to this optimized genetically engineered construct as acoustic reporter gene 1 or *arg1*.

To confirm that the ultrasound signal from *arg1*-expressing cells is due to the presence of gas vesicles, we applied acoustic pulses with amplitudes above the critical collapse pressure of the gas vesicles[20]. In purified samples, this resulted in the immediate collapse of these protein nanostructures and dissolution of their gas contents, eliminating ultrasound contrast[18,20]. As expected, the application of high-pressure pulses made cells expressing *arg1* invisible to ultrasound (Fig. 1d). The ability of ARG-based contrast to be erased *in situ* is used throughout this study to confirm the source of acoustic signals and subtract background.

arg1 expression resulted in gas vesicle contents of 9.4 ± 0.4 mg g^{-1} *E. coli* ($n = 3$, mean ± s.e.m.), corresponding to approximately 100 gas vesicles per cell. These nanostructures occupy roughly 10% of the intracellular space. Acoustically silent cells expressing the *B. megaterium* gene cluster produced a similar quantity of gas vesicle proteins (9.7 ± 1.5 mg g^{-1}, $n = 3$), underscoring the importance of genetic engineering in producing intracellular nanostructures with the appropriate size and shape to be detected by ultrasound. A fraction of *arg1*-expressing cells was buoyant in aqueous medium (Extended Data Fig. 2a, b), suggesting that gas vesicles occupy more than 10% of their volume. However, the expected buoyant force on these cells, even at much higher expression levels, is weak compared to other forces such as flagellar thrust (Supplementary Table 1).

To determine the detection limit of ARG-expressing cells, we imaged a concentration series of *E. coli* transformed with *arg1* (Fig. 2a). Cells at concentrations as low as 5×10^7 cells ml^{-1} produced a detectable signal (Fig. 2a, b). This equates to a roughly 0.005% volume fraction, or approximately 100 cells per voxel based on cubic voxel dimensions of 100 μm. This sensitivity should be sufficient for many *in vivo* scenarios[21]. Furthermore, bacteria enriched for buoyancy before imaging provide a 2.4-fold higher signal (Extended Data Fig. 2c, d), suggesting that sensitivity could be improved further by optimizing ARG expression.

To test whether ARGs could provide a read-out of state-dependent genetic pathways, we placed *arg1* under the control of a promoter

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