Synthetic Biology

Ultrasound imaging of gene expression in mammalian cells

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The study of cellular processes occurring inside intact organisms requires methods to visualize cellular functions such as gene expression in deep tissues. Ultrasound is a widely used biomedical technology enabling noninvasive imaging with high spatial and temporal resolution. However, no genetically encoded molecular reporters are available to connect ultrasound contrast to gene expression in mammalian cells. To address this limitation, we introduce mammalian acoustic reporter genes. Starting with a gene cluster derived from bacteria, we engineered a eukaryotic genetic program whose introduction into mammalian cells results in the expression of intracellular air-filled protein nanostructures called gas vesicles, which produce ultrasound contrast. Mammalian acoustic reporter genes allow cells to be visualized at volumetric densities below 0.5% and permit high-resolution imaging of gene expression in living animals.

The study of cellular function in the context of intact living organisms is a grand challenge in biological research and synthetic biology (1). Addressing this challenge requires imaging tools to visualize specific cells in tissues ranging from the developing brain to tumors, and to monitor gene- and cell-based therapeutic agents in vivo (2). However, most common methods for imaging cellular processes such as gene expression rely on fluorescent or luminescent proteins, which have limited performance in intact animals because of the poor penetration of light in biological tissue (3, 4). By contrast, ultrasound easily penetrates most tissues, enabling deep, noninvasive imaging with excellent spatial and temporal resolution (~100 μm and ~1 ms, respectively) (2, 5). These capabilities, along with its safety, portability, and low cost, have made ultrasound a widely used technology in biomedicine. Despite these advantages, to date, ultrasound has played a relatively small role in cellular imaging owing to the lack of appropriate genetically encoded reporters.

Recently, biomolecular contrast agents for ultrasound were introduced based on gas vesicles, air-filled protein nanostructures that evolved in certain waterborne bacteria and archaea to provide cellular buoyancy (6, 7). Gas vesicles comprise a 2-nm-thick protein shell enclosing a gas compartment with dimensions on the order of 100 nm. The acoustic impedance mismatch between their gas interior and surrounding aqueous media allows gas vesicles to strongly scatter sound waves and thereby serve as ultrasound contrast agents (8–12). In their native organisms, gas vesicles are encoded by clusters of 8 to 14 genes, including one or two primary structural proteins, and several other essential genes encoding putative assembly factors or minor shell constituents.

The use of gas vesicles as reporter genes requires the heterologous expression of their cognate multigene operon in a new cellular host, ensuring proper transcription and translation of each gene, functional folding of each corresponding protein, and appropriate stoichiometry and colocalization of the constituents for gas vesicle assembly. Recently, a genetic engineering effort succeeded in expressing gas vesicles as acoustic reporter genes (ARGs) in commensal bacteria, allowing their imaging in the mouse gastrointestinal tract (33). If ARGs could be developed for mammalian cells, then this would enable the study of how such cells develop, function, and malfunction within the context of model organisms and enable the in vivo imaging of mammalian cells engineered to perform diagnostic or therapeutic functions (14–16). However, developing ARGs for mammalian cells represents an even greater synthetic biology challenge because of the differences in transcription, translation, colocalization, and protein folding between prokaryotes and eukaryotes (17–19). To our knowledge, no genetic operon larger than six genes has been moved between these domains of life (20).

Here, we describe the expression of ARGs in mammalian cells to enable ultrasound imaging of mammalian gene expression. To identify a set of genes capable of encoding gas vesicle assembly in mammalian cells, we synthesized individual gas vesicle genes from three different microbial species using codons optimized for human expression, cloned each gene into a separate monocistronic plasmid, and transiently cotransfected mixtures of the genes from each species into human embryonic kidney (HEK) 293T cells (Fig. 1A). After allowing 72 hours for protein expression, we gently lysed the cells (~2 × 10⁶ cells per sample) and centrifugated the lysate to enrich for buoyant particles, which would include any gas vesicles. The top fraction of the centrifugated lysate was then screened for gas vesicles using transmission electron microscopy (TEM). These experiments took advantage of the intrinsic stochasticity of transient cotransfection, in terms of the ratios of genes and the overall DNA quantity delivered to each cell, to simultaneously sample a broad range of gene stoichiometries and expression levels without prior knowledge of parameters leading to gas vesicle formation.

The cotransfection of the gas vesicle genes from Halobacterium salinarum and Anabaena flos-aquae did not lead to the formation of detectable gas vesicles. However, the cotransfection of nine gas vesicle-encoding genes from Bacillus megaterium (Fig. 1B) resulted in the production of unmistakable gas vesicles, as evidenced by their appearance in TEM images (Fig. 1C). The 9 genes originate from an eleven-gene B. megaterium gene cluster previously used to express gas vesicles in Escherichia coli (33, 21), with the exception of GvpR and GvpT, which were found to be unnecessary for gas vesicle formation (fig. S1).

Using the nine genes identified in our stochastic screen, we set out to construct a polycistronic mammalian operon for consistent gas vesicle expression by joining these genes using the viral cotranslational self-cleavage peptide P2A (22). Having determined that all genes except GvpB could tolerate P2A peptide additions (fig. S2 and table S1), we constructed a polycistronic plasmid containing the eight P2A-tolerant gas vesicle genes connected by P2A sequences, and cotransfected it into HEK293T cells together with a plasmid encoding GvpB. Unfortunately, this did not result in the production of gas vesicles. We hypothesized that one or more of the genes in our polycistronic plasmid was expressed at an insufficient level, and used a complementation assay to identify GvpI, GvpF, GvpG, GvpL, and GvpK as bottleneck genes (fig. S3). This led us to construct a polycistronic “booster” plasmid containing these five genes, ordered to minimize P2A modifications to GvpI and GvpK, which were found to be most limiting. The cotransfection of the booster plasmid, together with the two plasmids above (Fig. 1D), enabled robust expression of gas vesicle reporters (Fig. 1E). We named this set of three genetic constructs ‘mammalian acoustic reporter genes,’ or mARGs. After establishing polycistronic constructs for mammalian gas vesicle assembly, we used an integrase (23, 24) to incorporate them into the cellular genome for stable expression under a doxycycline-inducible TRE3G promoter, with fluorescent proteins added to each construct as transfection indicators (Fig. 2A). We transfected these plasmids into HEK293T-tetON cells and used flow cytometry to sort cells according to the expression level of each fluorescent reporter. We found that the cell population combining the strongest expression of each construct
produced the largest number of gas vesicles (Fig. 2B and fig. S4, A to D). To ensure that mARG expression was not limited to HEK293 cells, we also transfected Chinese hamster ovary cells (CHO-K1) and obtained similar results (fig. S4, E to G).

To generate a stable monoclonal cell line expressing mARGs for detailed analysis, we sorted individual high-expression HEK293-tetON cells for monoclonal growth (Fig. 2C), producing 30 cell lines, which we screened for viability, fluorescence, and gas vesicle formation (Fig. 2D and table S2). The number of gas vesicles per cell was then estimated from TEM images, and a cell line yielding the largest quantity of gas vesicles was selected and named mARG-HEK. When induced for 72 hours with doxycycline (1 μg/ml) and 5 mM sodium butyrate (to reduce epigenetic silencing), this cell line produced on average 45 gas vesicles per cell (Fig. 2E). Using thin-section TEM, gas vesicles could clearly be seen in the cytosol of individual mARG-HEK cells (Fig. 2F). From TEM images of cell lysates, we measured the average dimensions of gas vesicles produced in this cell line to be 64 ± 12-nm wide (standard deviation [SD], 257 ± 121-nm long (SD, 257 ± 1282 gas vesicles), with some reaching lengths >1 μm (aspect ratios >30) (Fig. 2, G and H). This corresponds to an average gas vesicle volume of 0.605 attoliters. Together, the 45 gas vesicles expressed in an average mARG-HEK cell are expected to occupy just 0.0027% of the cell’s cytosolic volume.

The expression of gas vesicles did not change the gross morphology of mARG-HEK cells (Fig. 2I) and was nontoxic, as determined by three different assays (Fig. 2J), compared with a similarly prepared control cell line (mCherry-HEK) (fig. S5, A and B). During a 6-day coculture, mARG-HEK cells showed only a minor growth disadvantage compared with mCherry-HEK cells (Fig. 2K). As expected, both engineered cell lines grew more slowly than wild-type HEK293T cells (fig. S6).

Having engineered mARG-HEK cells, we sought to image their expression of acoustic reporters with ultrasound. Gas vesicles encoded by the B. megaterium gene cluster are expected to produce linear ultrasound scattering (27). However, because mammalian cells themselves also produce substantial linear contrast, detecting gas vesicles expressed in such cells using linear methods is challenging. To enable more selective imaging of mARG expression, we took advantage of the ability of gas vesicles to collapse irreversibly above specific ultrasound pressure thresholds (8, 9, 13, 21). A switch in the incident ultrasound pressure from below to above such a threshold results in a strong transient signal from the gas vesicles, which decays to a lower level in the next ultrasound frame owing to immediate dissolution of their gas contents and the elimination of ultrasound scattering (Fig. 3, A and B). Meanwhile, background tissue scattering rises with the increase in incident pressure and remains constant at the new level. Thus, images formed by taking the difference in signal between the collapsing and postcollapse frames reveal specifically the presence of gas vesicles.

We implemented this collapse-based imaging approach using an amplitude modulation pulse sequence (10), which we found to provide the best cancellation of non–gas vesicle signals. When hydrogels containing mARG-HEK cells were imaged with this technique at 18 MHz, they were easily distinguishable from mCherry-HEK controls on the basis of their contrast dynamics (Fig. 3C). Critically, although this imaging paradigm requires the collapse of gas vesicles inside cells, this does not affect cell viability (Fig. 3D).

To determine whether mARGs can faithfully monitor circuit-driven gene expression (25, 26), we measured the dynamic ultrasound response of mARG-HEK cells under the control of a doxycycline-inducible promoter (Fig. 3E). After induction with 1 μg/ml doxycycline, the cells showed a gradual buildup of ultrasound signal, with clear contrast appearing on day 2 and increasing over the next 4 days (Fig. 3F). These kinetics are similar to those observed with fluorescent indicators (fig. S7A). When the gene circuit was driven using a range of inducer concentrations, the ultrasound contrast followed the expected transfer function of the promoter (Fig. 3G and fig. S7B).

To determine how sensitively mARG-expressing cells could be detected in a mixed cell population, we combined mARG-HEK cells with mCherry-HEK cells at varying ratios. We were able to detect the presence of mARG-expressing cells in these mixtures down to 2.5% of total cells (Fig. 3H), corresponding to <0.5% volumetric density, or about three cells or 135 gas vesicles per voxel with dimensions of 100 μm. A similar voxel-averaged concentration of gas vesicles was detectable in a monoculture of mARG-HEK cells induced to express 1.4 ± 0.6 gas vesicles per cell (fig. S8).

In many imaging experiments, the output of a gene circuit is read out only once. However, in some cases, it may be desirable to track gene expression over time. We therefore tested whether mARG-expressing cells in which the gas vesicles have been collapsed during imaging could reexpress these reporters to allow additional imaging. mARG-HEK cells cultured in a nutrient-supported hydrogel produced clear ultrasound contrast 3 days after induction and were able to reexpress their acoustic reporters over three additional days (Fig. 3, I and J).

Having engineered mammalian cells to stably express gas vesicles and characterized their ability to produce ultrasound contrast in vitro, we next tested the ability of mARG expression to be visualized in vivo with high spatial resolution. We formed model tumor xenografts in immunocompromised mice by inoculating mARG-HEK cells in Matrigel subcutaneously in their left flanks (Fig. 4A). In the same mice, the right flanks were inoculated with mCherry-HEK control cells. We induced reporter gene expression in both tumors for 4 days with systemic injections...
Fig. 2. Formation, properties, and nontoxicity of gas vesicles in cells with genome-integrated mammalian ARGs. (A) Schematic of mARG constructs used for genomic integration into cells with the piggyBac transposase system. ITR, inverted terminal repeat; ChhGI, chicken b-globin insulator; GFP, emerald green fluorescent protein; BFP, enhanced blue fluorescent protein 2. (B) Representative TEM image of buoyancy-enriched lysate from HEK293-tetON cells transfected with the constructs in (A) and sorted for high expression of all three operons. (C) Fluorescence-activated cell sorting of HEK293-tetON cells transfected with the constructs in (A). Red circles denote individual cells selected by sorting to form monoclonal cell lines. (D) Selection process for monoclonal cell lines, including assays for viability, fluorescence intensity, and gas vesicle yield. (E) Number of gas vesicles expressed by monoclonal HEK293-tetON cells after 72 hours of induced expression, as counted in lysates using TEM. Bar represents the mean and the shaded area represents SEM (n = 3 biological replicates, each from two technical replicates). (F) Representative TEM image of a 60-nm section through an mARG-HEK cell showing an angled slice through two bundles of gas vesicles in the cytosol. (G) Representative TEM image of gas vesicles purified from mARG-HEK cells. (H) Size distribution of gas vesicles expressed in mARG-HEK cells. The mean and SD of both distributions is illustrated as a circle and with error bars (n = 1828 gas vesicles). (I) Phase-contrast images of mARG-HEK and mCherry-HEK cells 72 hours after induction with 1 mg/ml doxycycline and 5 mM sodium butyrate. (J) Cell viability of mARG-HEK cells relative to mCherry-HEK cells after 72 hours of gene expression. Error bars indicate SEM. (K) Fraction of mARG-HEK cells in coculture with mARG-mCherry cells seeded in equal numbers over 6 days of gene expression (n = 3 biological replicates, each from four technical replicates, with darker symbols showing the mean). Scale bars in (B), (F), (G) represent 500 nm; scale bar in (I) represents 20 μm.
Fig. 3. Ultrasound imaging of mammalian gene expression in vitro. (A) Illustration of the collapse-based ultrasound imaging paradigm used to generate gas vesicle–specific ultrasound contrast from mARG-expressing cells. (B) Representative non-linear signal recorded during a step change in the incident acoustic pressure, from 0.27 MPa in the white-shaded region to 1.57 MPa in the gray-shaded region. (C) Representative collapse and postcollapse ultrasound images of mARG-HEK and mCherry-HEK cells acquired during this ultrasound imaging paradigm and their difference, indicating gas vesicle–specific contrast. (D) Cellular viability after being insonated under 3.2-MPa acoustic pressures, as measured using the MTT assay. (E) Schematic of a chemically inducible gene circuit with mARG expression as its output. All three mARG cassettes in mARG-HEK cells are under the control of the doxycycline-inducible TRE3G promoter (TRE), with expression triggered by incubation with doxycycline. (F) Representative ultrasound images and contrast measurements in mARG-HEK cells as a function of time after induction with 1 µg/ml doxycycline and 5 mM sodium butyrate ($n=6$ biological replicates, with the darker dots showing the mean). (G) Representative ultrasound images and contrast measurements in mARG-HEK cells as a function of doxycycline induction concentrations. Cells were allowed to express gas vesicles for 72 hours in the presence of 5 mM sodium butyrate ($n=6$ biological replicates, with the darker dots showing the mean). (H) Schematic and representative ultrasound images from mARG-HEK cells in Matrigel reexpressing gas vesicles after acoustic collapse. Cells were induced with 1 µg/ml doxycycline and 5 mM sodium butyrate for 72 hours before and after 3.2-MPa acoustic insonation. Ultrasound images were acquired after an additional 72 hours in culture after collapse. (J) Ultrasound contrast in mARG-HEK and mCherry-HEK cells after initial expression, after collapse, after reexpression, and after second collapse ($n=7$ biological replicates, with the darker dots showing the mean). GV, gas vesicles. All scale bars represent 1 mm.
of doxycycline and sodium butyrate (Fig. 4B). We expected these nascent tumors to be mostly vascularized at their perimeter, resulting in the strongest inducible gene expression at the tumor periphery (Fig. 4A). Ultrasound, with its sub-100-μm spatial resolution (at 18 MHz), should be able to discern this gene expression pattern, whereas attaining such resolution would be challenging with optical techniques.

After 4 days of induction, we observed clear ultrasound contrast in the flank inoculated with mARG-HEK cells, and this was absent from the contralateral side (Fig. 4, C and D). As expected, the pattern observed with ultrasound revealed mARG expression at the perimeter of the tumor, whereas the core remained dark, and imaging of adjacent ultrasound planes revealed that this pattern of gene expression persisted across the tumor mass (Fig. 4E and fig. S9).

The ultrasound-observed spatial distribution of gene expression was consistent with the low vascularity in the tumor core, as observed with Doppler ultrasound (fig. S10). The peripheral gene expression pattern was confirmed with subsequent histological examination of the tissue (Fig. 4F and fig. S11). In comparison, our in vivo fluorescence images just showed the presence of signal somewhere in the tissue and not its precise distribution (Fig. 4G). These results, which were consistent across five animals (fig. S12A), demonstrate that mARGs enable gene expression imaging in vivo and highlight the ability of ultrasound to visualize intricate patterns of gene expression noninvasively. We imaged three of the animals again after an additional 4 days to look for reexpression of the collapsed gas vesicles and observed ultrasound contrast in each case (fig. S12B).

Our results establish the ability of an engineered genetic construct encoding prokaryote-derived gas vesicles to serve as a mammalian reporter gene for ultrasound, providing the ability to monitor cellular location and function inside living organisms. mARGs provide many of the capabilities associated with established genetically encoded optical reporters, including imaging cellular dynamics by promoter-driven expression and mapping cellular populations in complex samples. Whereas optical reporter genes mainly provide these capabilities in culture and surgically accessed tissues, mARGs enable gene expression to be resolved noninvasively in vivo.

Although the genetic constructs described in this work should be immediately useful in a variety of contexts, considerable scope exists for further optimization to make ARGs as widely useful as green fluorescent protein (GFP) (5, 12). For example, accelerating mARG expression beyond the day-scale kinetics shown in this study and developing sensitive imaging paradigms that do not require gas vesicle collapse would enable the imaging of more dynamic cellular processes. In addition, although this study demonstrated essential mARG functionality with clonally selected cell lines, the expression of mARGs in primary cells, their delivery to endogenous cells by viral vectors, and their expression in transgenic...
animals would greatly expand the utility of this technology. To facilitate such uses, it would be helpful to further condense the mARG constructs. For example, genes could be consolidated into fewer clusters, and preliminary experiments show that gvpB can be combined with the eight-gene polycistron encoding gcuv-gcuV through an internal ribosome entry sequence (IRES) (fig. S13). In addition, the total length of the coding sequence contained in mARG could be reduced from 7.6 to 4.8 kb by eliminating the need for redundant booster genes, relying instead on sequence contained in mARG could be reduced. Further optimization of mARG genetic constructs is also needed to reduce epigenetic silencing and metabolic burden (27–29). Just as the engineering of GFP over many years yielded brighter and more colorful reporters enabling new uses of fluorescence microscopy, further engineering of the genetic constructs comprising mARGs would help cellular ultrasound penetrate and enable new areas of mammalian biology and biomedicine.

REFERENCES AND NOTES
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Competing interests: A.F., G.H.H., D.P.S., and M.G.S. are inventors on U.S. patent application 15/663,635 submitted by the California Institute of Technology that covers mammalian acoustic reporter genes. A.F., R.W.B., and M.G.S. are inventors on U.S. patent application 15/663,635 submitted by the California Institute of Technology that covers bacterial acoustic reporter genes.

Data and materials availability: Plasmids encoding GvpB, GvpNFLKJU, and GvpFGLKJ are available from M.G.S. under a material agreement with the California Institute of Technology. The mARG genetic construct will be deposited with Addgene at the time of manuscript publication. Raw data are available from the authors upon reasonable request.

SUPPLEMENTARY MATERIALS
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Figs. S1 to S13
Tables S1 to S5
References (30–31)
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Sounding out mammalian cells

Live cell imaging allows us to observe cellular processes in real time. Most methods rely on light, and the poor penetration of light into tissues limits their application. Ultrasound penetrates tissues, and cellular reporters that respond to ultrasound have been developed recently. These reporters are air-filled protein structures that provide buoyancy in the bacteria they are derived from, but when surrounded by a fluid medium, they reflect sound waves. Farhadi et al. achieved expression from multiple genes to create these complex structures in mammalian cells. In addition to optimizing reporter production and detection, they visualize cells in a proof-of-principle experiment in mouse tumor xenografts.

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