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Beyond the Green Fluorescent Protein: Biomolecular Reporters for Anaerobic and Deep-Tissue Imaging

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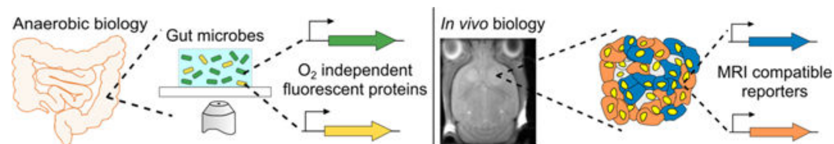
Abstract

Fluorescence imaging represents cornerstone technology for studying biological function at the cellular and molecular levels. The technology's centerpiece is a prolific collection of genetic reporters based on the green fluorescent protein (GFP) and related analogs. More than two decades of protein engineering have endowed the GFP repertoire with an incredible assortment of fluorescent proteins, allowing scientists immense latitude in choosing reporters tailored to various cellular and environmental contexts. Nevertheless, GFP and derivative reporters have specific limitations that hinder their unrestricted use for molecular imaging. These challenges have inspired the development of new reporter proteins and imaging mechanisms. Here, we review how these developments are expanding the frontiers of reporter gene techniques to enable nondestructive studies of cell function in anaerobic environments and deep inside intact animals—two important biological contexts that are fundamentally incompatible with the use of GFP-based reporters.

Graphical Abstract



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INTRODUCTION

Genetically encoded reporters such as the green fluorescent protein (GFP) provide one of the most powerful techniques for molecular imaging in living systems. However, GFP and related proteins strictly rely on oxygen to activate fluorescence, which makes these reporters unreliable in anaerobic conditions.[1–3] Furthermore, as biological systems scale in complexity from cultured cells and transparent organisms to warm-blooded animals, the utility of GFP (and optical reporters in general) is increasingly curbed due to inadequate penetration of light through deep tissues.[4–6] As a result, GFP-based reporters come up short against the capabilities required for studying biological function in anaerobic cell cultures and within opaque animals. This is a serious limitation as anaerobic organisms and animal models have immense value in both basic research and emerging biotechnologies such as sustainable biomanufacturing, cellular therapies, and cell-based diagnostics.[7–24] To effectively tackle these challenges, new classes of genetic reporters have been introduced that employ various modalities—fluorescence, magnetic resonance (MRI), ultrasound, and photoacoustics—to visualize cellular function in the aforementioned *in vitro* and *in vivo* environments where GFP-based fluorescence is stifled.[25–27] While these reporters have yet to attain the pervasiveness of GFP, they represent a major advance toward breaking long-standing barriers in molecular imaging. Importantly, the study of complex cellular communities that thrive in low-oxygen milieu (e.g., the gut microbiota, which is comprised largely of anaerobic microbes) would benefit tremendously from the availability of these nontraditional reporter mechanisms that can be used synergistically to interrogate biological function across cellular and organismal scales.[28] In this Topical Review, we summarize recent developments in two such classes of biomolecular reporters—oxygen-independent fluorescent proteins and tissue-penetrant MRI reporters—that respectively enable biological function to be visualized in the context of anaerobic cells and intact research animals. In addition, we identify key avenues where innovative engineering solutions are needed to empower these nontraditional reporters so as to parallel or even rival the capabilities of GFP.

LOV-BASED FLUORESCENT PROTEINS: KEEPING CELLS GLOWING WHEN OXYGEN RUNS OUT

Reporters derived from GFP have limited utility for studying gut microbes, hypoxic tumors, and other systems that thrive in low oxygen conditions.[29–33] To this end, efforts to build oxygen-independent fluorescent proteins have led to the discovery of biomolecular reporters derived from flavin-binding photoreceptors known as light, oxygen, and voltage (LOV) sensing proteins.[34,35] LOV proteins are photochemists by cellular profession. Using flavin as their photoactive cofactor, wild-type LOV proteins convert blue light excitation into conformational rearrangements to modulate effector functions such as kinase activity and DNA binding.[36] Wild-type LOV is dimly fluorescent because the energy from photoexcitation is expended to coordinate electronic and structural changes in the protein. However, by mutating a key photoactive cysteine residue into alanine, it is possible to impair this photochemistry, thereby turning the resultant Cys → Ala mutant fluorescent.[37,38] A hallmark of this fluorescence mechanism is that light emission is independent of oxygen (Figure 1A), spectral properties being largely determined by interactions between the LOV

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protein and the noncovalently bound flavin cofactor. Building on this foundation, nearly 20 LOV based fluorescent reporters have been derived from bacteria, plants, and environmental metagenomic libraries.[37–42] To potentiate their utility as reporter proteins, engineering techniques such as DNA shuffling and site saturation mutagenesis have been applied to increase photostability, thermal tolerance, and quantum yield.[43–45] Further more, using genome mining, we recently introduced new algal LOV reporters that are characterized by improved molecular brightness, photostability, broad pH range, and thermal stability.[46] However, all known LOV proteins share conserved spectral traits characterized by broad excitation and emission spectra with peaks at 450 and 495 nm, extinction coefficient of ~14 mM^{-1} cm^{-1}, and quantum yields in the 0.17–0.51 range.[34,35,40,42]

LOV reporters have found use for imaging a broad diversity of anaerobic bacteria, fungi, and hypoxically cultured mammalian cells.[47–59] These applications have ranged from tracking horizontal gene transfer and cell division in anaerobic environments to screening promoters for metabolic engineering and genetic circuit designing in anaerobes.[51,52,60–64] Other applications have harnessed the small size of LOV reporters (~13 kDa, half that of GFP) to translationally tag proteins in scenarios where the comparatively larger steric footprint of GFP interferes with protein function.[38,55,65,66] In addition, several LOV reporters display remarkable pH stability (pK_a ~ 3 for EcFbFP and CreiLOV), which has been exploited to develop pH sensors based on Förster resonance energy transfer (FRET) between LOV and a pH-sensitive GFP variant.[46,67] Aside from FRET, LOV-based biosensors have also been constructed through the incorporation of unnatural amino acids in the flavin binding pocket to selectively modulate fluorescence via photoinduced electron transfer between the amino acid and flavin.[68]

KEY CHALLENGES WITH EXISTING LOV BASED FLUORESCENT REPORTERS

As with any technology in its infancy, the existing suite of LOV-based reporters comes with limitations as well as exciting opportunities for future research. First and foremost, even the brightest LOV reporters are considerably dimmer than GFP, achieving only 10% of GFP fluorescence upon expression in cells.[2,46] Given that cellular brightness of any reporter depends on multiple factors such as protein stability, solubility, and effective fluorescent fraction, it is important for future engineering efforts to pursue integrated improvements in spectral and biochemical properties of LOV proteins. In principle, it should be possible to increase cellular fluorescence at least 4-fold by concurrently maximizing quantum yield (2-fold increase theoretically possible) and solubility (soluble fraction in *E. coli* for most LOV reporters <50%, unpublished data from our lab) in the brightest available LOV reporters. This could be achieved either through rational structure guided mutagenesis or through directed evolution techniques. A second challenge stems from the lack of variously colored LOV reporters, particularly ones that are sufficiently red-shifted to avoid overlapping with cellular autofluorescence. A recent theoretical study suggested the possibility of red-shifting LOV reporters using specific mutations, but these predictions were contradicted by follow-up experiments.[69–71] To our knowledge, efforts to engineer spectral shifts in the existing LOV repertoire using directed evolution have also proved unsuccessful. Going by the

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tremendous impact of multicolored GFP variants,[72] it is clear that innovative approaches to diversify the LOV color palette should be a major focus of future research in this area. A final unexplored avenue relates to the possible effects exerted on a cell's endogenous flavin pool due to overexpression of LOV proteins. LOV reporters bind flavins with moderately strong affinity (K_d ≈ 169–178 nM, unpublished data from our lab), which is sufficient to deplete free reserves of cellular flavin, estimated to be 4 μM in *E. coli* and mammalian cells.[73–76] It has therefore been suggested (albeit not experimentally studied) that flavin sequestration by LOV reporters leads to an increase in flavin biosynthesis via feedback mechanisms.[40] Could LOV reporters perturb flavin homeostasis sufficiently to affect cell physiology? Alternatively, could cellular fluorescence of LOV proteins be enhanced by augmenting flavin biosynthesis? Rigorous benchmarking of LOV reporters against complementary readouts of gene expression (e.g., lacZ based colorimetric assays) in different environments, across multiple cell types, and varying intracellular flavin levels should help address these questions.

ALTERNATIVE BIOMOLECULAR REPORTERS FOR ANAEROBIC IMAGING

In addition to LOV proteins, at least three distinct classes of biomolecular reporters hold promise for further expanding the low-oxygen imaging toolbox. The first class consists of heme-based reporters, specifically phytochromes and UnaG, which generate fluorescence upon binding end-products of heme degradation such as biliverdin and bilirubin (Figure 1B).[77,78] Notably, UnaG and phytochromes have been used to engineer several fluorescent protein biosensors (albeit, yet to be demonstrated in anaerobic systems), which could be useful for probing kinase activity, calcium signaling, redox, protein–protein interactions and other aspects of cell function in varying oxygen settings.[79–81] However, one key challenge with using heme-based reporters is that bilirubin and biliverdin are not native to all cell types —for instance, bacteria do not typically synthesize either heme metabolite.[3] In such cases, the chromophores need to be exogenously delivered, which is hindered by the poor permeability and limited aqueous solubility of heme compounds. As a workaround, phytochromes have been used in conjunction with heme oxygenase to directly synthesize biliverdin in cells via enzymatic breakdown of heme.[82–84] However, the enzymatic conversion of heme to biliverdin requires oxygen, which makes this approach impractical in anaerobes. The second class of low-oxygen compatible reporters consists of fluorogenic proteins (e.g., derivatives of photoactive yellow protein or PYP) and RNA aptamers engineered to bind synthetic small molecule dyes (e.g., hydroxybenzylidene imidazolinone, coumarin) and activate fluorescence by suppressing fluorescence quenching intramolecular movements in the unbound dye.[85–92] Although PYP-based fluorogenic reporter proteins have not yet been demonstrated in anaerobic systems, an RNA-based reporter was recently engineered to develop an anaerobically compatible biosensor for detecting the cellular second messenger, cylic-di-GMP (Figure 1C).[88] While promising, PYP and aptamer-based reporters demand a steady supply of externally added fluorophores, which poses a potential problem in cell types where membrane permeability is low (e.g., Gram-negative bacteria) as well as in studies where intrinsic membrane permeability is affected by the experimental conditions (e.g., antibiotic use).[93–97] Finally, a new class of fluorescent reporters has been recently developed based on microbial opsins that bind retinal or synthetic analogs as their

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chromophore.[98,99] While this fluorescence mechanism is likely to be oxygen-independent (although yet to be demonstrated experimentally), the poor solubility of opsin-based fluorescent proteins restricts expression to the plasma membrane, which potentially limits their broad utility as biomolecular reporters.[98,99]

MOLECULAR IMAGING *IN VIVO*: REPORTER GENES FOR MAGNETIC RESONANCE IMAGING (MRI)

Fluorescent reporters fail to achieve the depth and coverage needed to comprehensively study biological function in the context of intact, opaque animals.[4,100–102] In contrast, MRI enables noninvasive tomographic imaging with excellent soft tissue contrast and spatiotemporal resolution. Small animal MRI scanners routinely acquire images of entire tissue sections at any depth *in vivo* and with a resolution of ~250 μm with acquisition lasting tens of seconds to minutes. Thus, MRI compatible reporter genes provide a promising approach for molecular imaging in living subjects. To this end, at least 25 proteins have been genetically encoded in cells and tissues to generate MRI contrast via at least 5 different mechanisms.[27,103] Although not discussed in this review, prominent *in vivo* reporter genes have also been developed based on non-MRI mechanisms—notably, bioluminescence (e.g., luciferase) and positron emission tomography (e.g., thymidine kinase). These reporting mechanisms provide complementary advantages to MRI such as ease-of-use and detection sensitivity, albeit with associated trade-offs in penetration depth (bioluminescence), spatial resolution, and need for external substrates.

METALLIC MRI REPORTER GENES

The dominant mechanism for engineering MRI reporters involves pairing a protein with a suitable paramagnetic metal ion ($Fe^{2+/3+}$, Gd^{3+}, Mn^{2+}) or metallic substrate to produce contrast by speeding up spin–spin (T_2) or spin–lattice (T_1) relaxation rates of magnetically polarized water protons.[103–105] MRI reporters that employ this mechanism include metal storage proteins (e.g., ferritins, bacterial encapsulins[106–113]), metallo-enzymes (e.g., cytochrome P450[114]), and metal transporters (e.g., transferrin receptor,[115,116] Timd2[117]). Importantly, ferritin and related reporters have been shown to generate T_2 contrast simply by accumulating endogenously available iron, thus avoiding a need for external metal delivery (Figure 2A). However, *in vivo* detection using ferritin-based proteins has often proved difficult due to inadequate magnetization in the cellular environment as well as interference from pathological conditions such as hemorrhage that produce identical signal changes to T_2 reporters.[104,118–121] These roadblocks can be overcome through the use of Gd^{3+} and Mn^{2+} based metalloproteins that produce T_1 weighted contrast (e.g., MntR,[122] DMT1,[123] Oatp1,[124] and ProCA32[125]). However, the challenge here lies in delivering Gd^{3+} and Mn^{2+} ions sufficiently and uniformly to target sites *in vivo*. Notably, exogenously delivered metals are known to distribute unevenly depending on structural heterogeneity (e.g., within a tumor microenvironment[126]) and physiological state (e.g., activated neurons selectively accumulate extracellular Mn^{2+} unlike resting neurons[127,128]). Furthermore, delivering metals to fortified tissues such as the brain might require sophisticated procedures for opening the blood brain barrier.[128–130] Despite these limitations, metallic MRI reporter genes have been used with

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some success for *in vivo* tracking of cells and cell-based therapies. Here, therapeutic cells (e.g., immune cells, stem cells, probiotic microbes) are transfected or transduced with a metal-based MRI reporter gene before transplanting the genetically labeled cells in an animal.[109,131–133] If labeling is done *ex vivo*, the cells can be preloaded with a sufficient concentration of metal ions to achieve maximum contrast. Using MRI, the migration and distribution of cell therapies can then be tracked noninvasively inside a living subject (Figure 2A). However, a fundamental challenge stems from the low sensitivity of MRI reporter genes, which limits *in vivo* detectability of transplanted cells except when delivered in large (often clinically unrealistic) concentrations. Finally, it is worth noting that any metal-based contrast mechanism is likely to interfere with natural metal homeostasis in cells and tissues, which circumscribes the safety window for using metallic MRI reporters.[119,121,134]

NONMETALLIC MRI REPORTER GENES

The above challenges have stimulated the development of metal-free MRI reporters, which leverage contrast mechanisms that avoid external substrates altogether. One such mechanism is chemical exchange saturation transfer (CEST), which is based on selective absorption of radiofrequencies by a contrast agent whose protons are chemically shifted from bulk water protons.[135,136] By carefully matching this chemical shift with kinetics of proton exchange between the contrast agent and bulk water, it becomes possible to transfer magnetization from the agent to water, thereby encoding MRI contrast without a need for metals.[137] Furthermore, by choosing contrast agents with different chemical shifts, multiple agents can be imaged in the same field of view, which provides a unique avenue for multiplexed MRI.[138] Robust CEST effect demands a sufficiently high reporter concentration to ensure an abundant supply of magnetized protons for exchanging with the substantially larger (~70 M) pool of intracellular water protons.[27,135,139] As a result, CEST-based reporters require an unusually large density of exchangeable protons, which is not available in most natural proteins. Successful CEST reporters have thus made use of synthetic polypeptides with hundreds of exchangeable protons (e.g., polylysine, polyarginine) and highly cationic proteins such as protamine and supercharged GFP.[140–144] Unfortunately, even at high expression levels, these reporters produce modest contrast (Figure 2B).[139,141,145] Another challenge is that the chemical shift between water and reporter protons is typically not large enough to avoid overlapping with endogenous sources of exchangeable protons from native proteins, lipids, and cellular metabolites.[146] Collectively, the aforementioned limitations lower overall detection sensitivity, which is one of the main challenges toward robust imaging using existing CEST-based reporter genes. To this end, an important engineering goal is the design of CEST reporters where the chemical shift of exchangeable protons is large enough (>5–6 ppm) to avoid overlapping with cellular background.

Recently, we introduced a new paradigm for genetically encoding MRI contrast without a need for metals or highly charged peptides.[147] Our approach makes use of specific water channels known as aquaporins to facilitate water exchange across the cell membrane. Water exchange across lipid membranes is typically constrained by high activation energy (>10 kcal/mol),[148] which lowers overall water diffusion in tissues by 5–6-fold compared to unrestricted diffusion.[149,150] By engineering cells to express aquaporins, it becomes possible to increase transmembrane water flux without affecting the cell's osmotic state. The

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resulting increase in diffusivity can be imaged using an MRI technique weighted to water diffusion. Using this approach, we were able to detect switchable gene expression in intracranial murine tumors engineered to express a human aquaporin known as AQP1 (Figure 2C).[147] Interestingly, *in vitro* experiments based on diffusion weighted imaging of AQP1 expressing cell lines established a detection limit of ~500 nM and demonstrated that micromolar concentrations of membrane AQP1 provided sufficient signal-to-noise for detecting down to 3×10^3 cells per MRI voxel.[27,147] These attributes make AQP1 considerably more sensitive compared to most MRI reporter genes. The increased sensitivity results from the rapid kinetics of water exchange (~10^9 water molecules/channel/s[151]), which allows low concentrations of AQP1 to effectively mediate exchange between the larger pools of intra- and extracellular water molecules. Shortly after this work, Brindle and colleagues reported a similar mechanism for producing MRI contrast using a urea transporter (UT-B) to exchange water across the plasma membrane.[145] Leveraging the high sensitivity of this contrast mechanism, the authors demonstrated detection of lentivirally transduced gene expression directly in brain parenchyma. An important challenge with diffusion-based MRI reporters relates to unambiguously resolving reporter signals from background in anatomical regions where water diffusivity is high—for example, necrotic lesions, cysts, and healthy tissues, which natively overexpress aquaporins (e.g., glial cells). Engineering aquaporins for faster water exchange and developing techniques to separate aquaporin-mediated changes in diffusion from background diffusivity are important avenues for future research in this area.

The latest addition to the repertoire of genetic MRI reporters is a unique class of gas filled protein nanostructures known as gas vesicles.[152] These ~10-aL-sized air-filled protein compartments perturb local magnetic gradients, which generates T_2 weighted contrast. Although biogenic, gas vesicles are complex, multigene reporters encoded by clusters of 8–14 genes in their native organisms.[100] Genetic synthesis of gas vesicles in non-native platforms was recently demonstrated.[153,154] However, MRI-based imaging of genetically encoded gas vesicles has thus far been feasible only in *E. coli* cells.[152]

CONCLUSIONS AND OUTLOOK

Despite innovative advances in reporter engineering, we are still missing clear go-to reporter genes that can be used with the ease and rigor of GFP for visualizing biology in anaerobic cells and intact living organisms. While LOV proteins are likely front runners for the former, concerted efforts are required to improve cellular brightness before LOV proteins can be routinely and reliably used as anaerobic reporters. Furthermore, as several anaerobes display high levels of autofluorescence (e.g., methanogens, anaerobic gut fungi), the need to introduce bathochromic shifts in the anaerobic reporter spectrum is immediate and paramount. To this end, photo-responsive proteins that make use of unconventional biogenic chromophores (apart from flavins and hemes) with genetically encodable (oxygen-independent) biosynthetic pathways could serve as new sources of fluorescent reporters. In contrast to fluorescence, a broader diversity of protein properties—paramagnetism, water diffusion, and air compartmentalization—have been employed to build MRI reporters. Of these, metal-based reporters are at a reasonably mature stage of development—but their dependence on metal cofactors curtails some of the key capabilities associated with genetic

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reporters. CEST reporters elegantly solve the problem of metal/substrate requirement albeit at the cost of reduced sensitivity and a limited reservoir of native proteins that can serve as building blocks for reporter engineering. Newly introduced diffusional reporters such as AQP1 and UT-B are sensitive metal-free contrast agents with homology to multiple naturally occurring channels. In future work, homologous channels could be mined for new reporters with faster water exchange (for improved signal-to-noise) and/or biochemically gated water flux, the latter providing a molecular template to guide sensor engineering. Finally, if further advances in gene expression are forthcoming, gas vesicles could emerge as a uniquely powerful class of reporter genes for background-free MRI.

Alongside reporter and sensor engineering efforts, there is a clear need for studies that make use of the aforementioned reporter gene techniques beyond proof-of-concept experiments. Only then will it be possible to recognize practical limits of various emerging reporter mechanisms as well as identify vital avenues for improvement. From this standpoint, the alliance of oxygen-inert fluorescent proteins and tissue-penetrant MRI reporters can provide a comprehensive molecular window into a gamut of low-oxygen pathophysio-logical processes such as bacterial response to antibiotics in anaerobic infections, effects of tumor hypoxia on treatment outcome, and gut microbiology. To this end, one exciting area of research where we envision the use of LOV reporters to effectively synergize with MRI-compatible reporter genes is in the study and engineering of intestinal microbes for diagnostic and therapeutic capabilities.[28] Here, LOV-based proteins could be first applied to design, build, and test new genetic parts and gene networks in the context of anaerobic cultures of intestinal microbes. Subsequently, MRI-based reporters could be used to study the location, performance, and intestinal transit of the engineered microbes in animal models of gut function and dysbiosis, thus effectively bridging cellular scale studies with whole organism level understanding of biological function (Figure 3).

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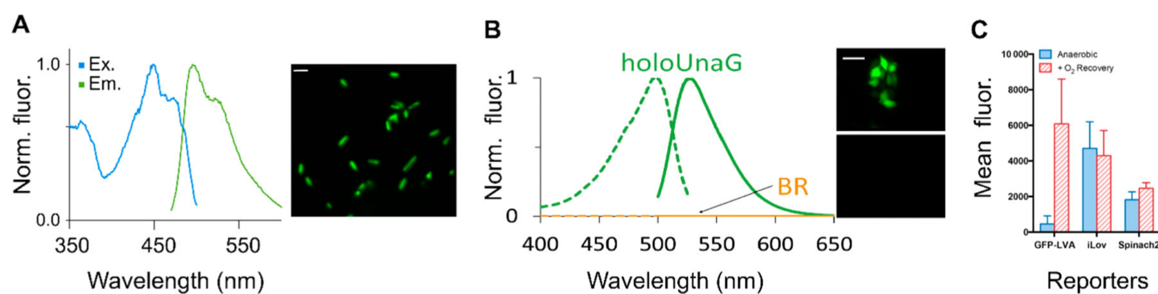

Figure 1.

Oxygen-independent biomolecular reporters. (A) Fluorescent proteins derived from LOV photoreceptors are characterized by 450 nm excitation and 495 nm emission. Anaerobically cultured *E. coli* cells expressing CreiLOV[46] can be readily detected using fluorescence microscopy.(B) UnaG emits green fluorescence by associating with a heme end-product (biliverdin), which enables imaging of HeLa cells in 0.1% hypoxia (top panel).[77] Under similar conditions, HeLa cells that express mCherry (bottom panel) are nonfluorescent.[77] (C) Spinach2 is a small molecule dye-binding aptamer that exhibits oxygen-independent fluorescence similar to iLOV, a LOV-based fluorescent reporter.[88] In contrast, GFP is nonfluorescent in anaerobic conditions and needs oxygen to activate fluorescence.[88]

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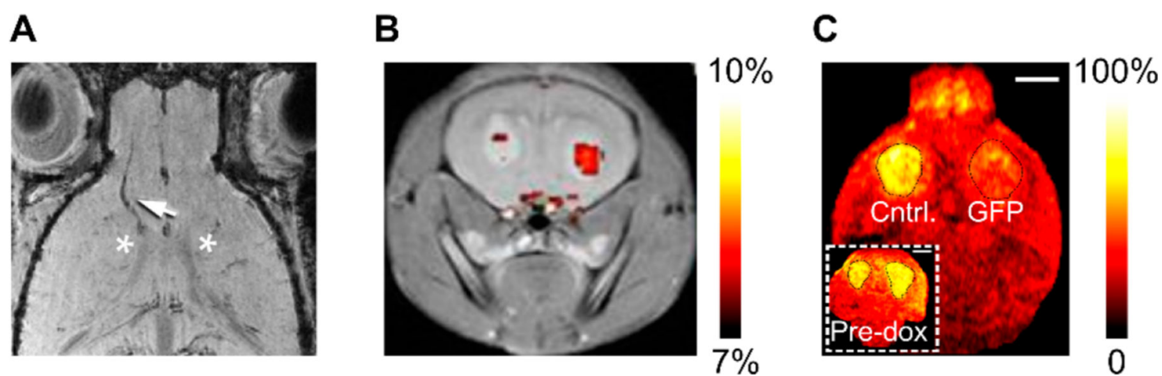

Figure 2.

Reporter genes for MRI. (A) Genetically encoded ferritin enables migrating neuroblasts to be tracked *in vivo*, as indicated by the white arrow.[109] (B) Charged polypeptides, in this case polylysine, can be used to visualize gene expression from a tumor specific promoter, using CEST.[141] (C) Aquaporins produce diffusion weighted MRI contrast, which can be used to dynamically monitor doxycycline (dox) induced changes in tumor gene expression.[147]

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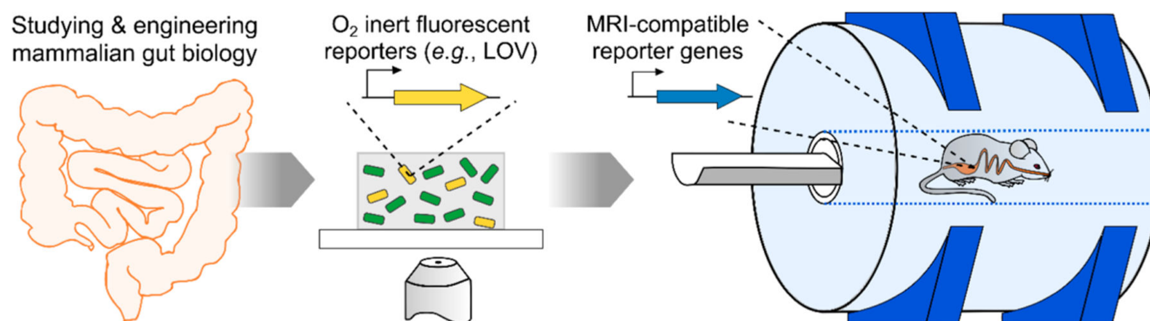

Figure 3.

Oxygen-independent fluorescent proteins can be used in conjunction with MRI reporter genes to provide a unique molecular window into gut biology across cellular and organismal scales.