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Review

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The regulation of reporter transgene expression for diverse biological imaging applications

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A common strategy in biological research is to use the expression of a reporter transgene to non-destructively visualize or measure a biological parameter or process that is otherwise not visible or readily detectable. Genetic reporters have greatly impacted practically all fields of biological research, from visualizing biology in bacteria and microbes, through plant biology, to preclinical research in higher eukaryotes. Fundamentally, many reporter gene assays are reliant upon the expression of a relatively small number of transgenes (e.g., green fluorescent protein (GFP) or firefly luciferase (Fluc)), however, by regulating their expression in different ways, it is possible to generate enormous diversity in the specific aspects of biology measured. For the purposes of this review, rather than focus on the non-invasive imaging modalities or reporter transgenes themselves (recently reviewed[1,2]), we will instead discuss how reporter transgene expression can be manipulated to generate a huge variety of biological readouts, with specific emphasis in the fields of preclinical oncology and neuroscience, now coming together in the nascent field of cancer neuroscience.

Researchers have access to a broad range of preclinical imaging modalities that can noninvasively measure different aspects of in vivo biology. These permit repeated assessment of biology within the same subject, enabling longitudinal analyses that show biological change over time, or noninvasive "before and after" measures following experimental perturbation or treatment with a therapeutic. Each imaging modality has comparative strengths and weaknesses, no single modality is best suited to address all types of biological questions. Therefore, the modality should be selected according to the nature of the question, according to whether sensitivity or resolution is most important, whether whole-body and deep-tissue scans are needed, or whether images at cellular or high temporal resolution are required.

A popular way to generate an imaging signal or boost signal intensity for nearly every imaging modality is to introduce the expression of a reporter transgene into the target cell population. These are genetically encoded elements that make it possible to noninvasively detect and measure aspects of in vivo biology that otherwise would not be visible. As reporter transgene labeling requires genetic modification of the target cell or tissue, their use in research has been mainly restricted to a preclinical and experimental context. A notable exception to this has been the use of reporter transgenes in clinical gene therapy research or cellular CAR-T cell therapy, whereby efficient genetic modification of the recipient cell is a key component of the overall therapeutic goal which must be measured to fully interpret treatment results[3].

In the most basic sense, reporter transgenes are expressed from a simple transcriptional unit, comprising a promoter region, the reporter transgene itself, and possibly an intron and poly-A tail (see Fig. 1). Despite this relative simplicity, reporter transgenes have been used to measure truly diverse aspects of preclinical biology. Depending on the actual reporter and associated modality, these readouts can range both in their scale, from microscopic to whole-body imaging, as well as in the specific nature of the biology measured. This review focuses on how both the regulation of reporter transgene expression and the method chosen to introduce it can generate diverse biological imaging readouts. We illustrate this from the aspect of preclinical oncology and neuroscience research; two fields of experimental biology that have exemplified many of the diverse biological applications of genetic reporters in research, now coming together in the exciting new area of cancer neuroscience. We acknowledge that this is an extensive topic and it is beyond the scope of this review to comprehensively cite all relevant papers. Rather, we support key points with one or two salient examples from the literature.

Popular reporter transgenes

Reporter transgenes have been developed for almost all mainstream imaging modalities (see Table 1). In general terms, they can be thought of as either generating or enhancing the signal or contrast of any given imaging modality, even permitting manipulation of the labeled cell[4]. Choosing which reporter to use is therefore dependent upon experimental need and the

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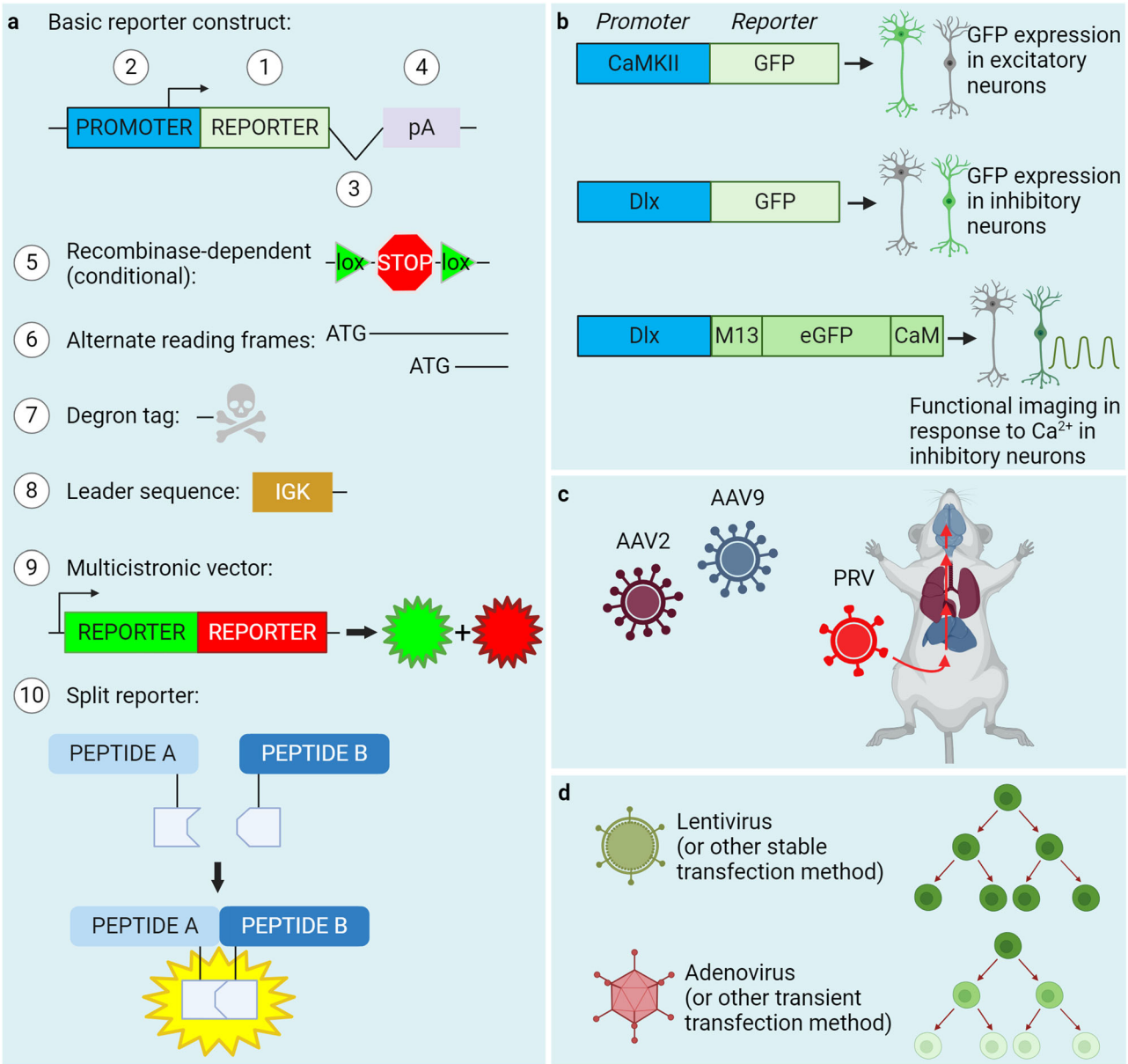

Fig. 1 | Common transgenic and vector-based strategies that influence transgene expression. a The basic architecture of an imaging reporter transgene expression construct (components 1–4). A synthetic splice site (3) and polyA sequence (4) are commonly utilized in many nonviral vectors to promote transgene expression level and RNA stability, but their inclusion may affect the performance of some viral vectors (e.g. lentivirus). Additional transgenic features can be added to this base unit to substantially broaden functionality. For example, a lox-stop-lox cassette (5) can be positioned between the promoter and transgene to make reporter expression Cre-dependent (a popular approach to achieve cell- or tissue-specific transgene expression (n.b. FLP/frt can be used as an alternative)). Many other transgenic sequences can also be positioned in-frame with the reporter to produce signal dependent on biological context. For example, alternate reading frames that are biology dependent (6) can be positioned upstream, or a degron peptide tag (7) fused to the N' or C' terminus of the reporter, to confer biology-specific expression. Alternatively, a leader sequence (8) can be added to place the reporter protein in the cell membrane or have it secreted. Additional reporters can be expressed from the same promoter either by fusing them, or positioning an IRES or 2A sequence (in-frame and the stop codon removed) between reporter coding sequences (9). Protein: protein interactions can be imaged by fusing complementary fragments of a split reporter, or paired FRET or BRET reporters to peptides of interest (10). Imaging signal is contingent upon both reporters being brought into immediate proximity (i.e. when the proteins that they are fused to bind). **b** Different promoter sequences can drive transgene expression in different neuron types, including excitatory (CaMKII) or inhibitory (Dlx) neurons. Fusing GFP to calcium sensing machinery (M13 and calmodulin (CaM)) allows cell-type specific monitoring of neuronal activity. **c** These different transgenes can be delivered either locally or systemically based on the viral serotype and tropism. For example, AAV9 has high tropism for neurons and cardiac tissue and can cross the blood brain barrier, while AAV2 is more selective for epithelial and retinal cells. Pseudorabies virus (PRV) can further be used to trace long-range polysynaptic neuronal connections given its unique ability to travel across several synapses along a neural circuit chain. By modifying promoters, transgene structure, or viral serotype, one can achieve cell-type specific expression of virtually any genetic cargo (depending on viral capsid packaging limits). **d** For the delivery and long-term expression of a reporter transgene into populations of dividing cells (e.g. cancer), it is important to use a vector that stably integrate into the host genome (e.g. lentivirus). Transient vectors (e.g. adenovirus) are useful for short-term studies or for imaging terminally differentiated/non-dividing cells, as expression levels drop significantly with each round of cell division. (Created in BioRender. Merrill, J. (2024) https://BioRender.com/r71u177).

Table 1 | Popular reporter transgenes

Reporter	Modality	Mechanism	Advantages	Disadvantages	Reviewed
Firefly or Renilla luciferase and variants	Optical: Bioluminescence	Enzymatic	Excellent sensitivity, low cost, high throughput. Large number of variant enzymes and custom-made substrates available	Signal is scattered and absorbed by overlying tissue. Relatively low image resolution, signal is surface-weighted	68
Green fluorescent protein (GFP) and variants	Optical: Fluorescence (whole body)	Photoexcitation followed by high quantum yield emission	Relatively low cost, large number of spectrally-distinguishable variants available	Spectrally-dependent tissue penetration, autofluorescence can affect SNR	69
	Optical: Fluorescence (intravital microscopy)	Photoexcitation followed by high quantum yield emission	Large number of spectrally-distinguishable variants available. Up to intracellular spatial resolution, up to millisecond temporal resolution	Sub-millimeter tissue depth and limited field-of-view. Some studies involving tissue-windows limit period of assessment.	70
	Optical: Fluorescence (light sheet)	Photoexcitation followed by high quantum yield emission	Imaging of whole organs or body	Requires optically cleared tissue	71
	Optical: Fluorescence (fusion proteins for chemical sensing and optical readout)	Photoexcitation followed by high quantum yield emission	Quantitative imaging corresponds to changes in signaling/chemicals. In vitro and in vivo application	Poor signal-to-noise ratio can make interpretation difficult. Spatial resolution can be limited.	29,37,38
Sodium iodide symporter (NIS), Herpes simplex virus – tyrosine kinase (HSV-tk)	PET/SPECT	Transporter, or enzymatic	Quantitative, excellent sensitivity, tissue-depth independent	Imaging probes are radioactive, cost can be high	72
Ferritin, Organic anion-transporting polypeptides (OATP)	MRI	Iron sequestration, or transporter	Excellent image resolution, signal independent of tissue-depth	Signal enhancement can be modest relative to other reporters, relatively expensive modality	73
Acoustic reporter genes (ARG)	Ultrasound	Gas-filled vesicle	Excellent image resolution, relatively low cost	Transgene is large and multi-component, limiting some gene delivery options. US not well-suited for whole-body scans	74
Tyrosinase, near-infrared fluorescent proteins (NIR FP)	Photoacoustic	Photon quenching, or photoexcitation followed by low quantum yield emission	Uniquely, both reasonable image resolution and sensitivity	Spatial resolution worsens with tissue penetration depth	75

properties of the imaging modality, but scanner access and the cost or availability of any associated imaging material (e.g. radiolabeled probes or imaging substrate) will also be key determinants.

The imaging signal from a reporter can be generated directly or indirectly. For example, fluorescent or light-absorbing proteins for FLI (fluorescence imaging) or PAI (photoacoustic imaging) respectively are direct, whereas enzymatic or membrane transporter proteins, such as firefly luciferase for BLI (bioluminescence imaging) or NIS (sodium iodide symporter) for SPECT (single photon emission computed tomography) or PET (positron emission tomography) are indirect. It should be noted that the signal intensity from an indirect reporter is more prone to be affected for reasons other than the biological parameter being measured. For example, cancer drugs may result in the elevation of drug efflux pump expression, consequently reducing bioavailable substrate, causing a reduction in signal unrelated to the intended biology measured. It is therefore important that researchers histologically validate such imaging readouts. In the context of BLI, a series of constructs have been developed with two spectrally distinct enzymes that metabolize the same imaging substrate. The intensity of one color changes according to the biological state of the cell, whereas the intensity of the other is "always on" and so can be used as an internal control to normalize the functional signal for any changes resulting from indirect effects on signal generation[5].

Of special mention, fluorescent reporters have had a big impact on both preclinical oncology and neuroscience research. They are available in many spectrally distinguishable colors (affording multiparametric imaging) and are sufficiently bright for microscopic imaging of tissues at cellular resolution in vitro or in vivo (via intravital microscopy), as well as on tissues ex vivo. Tissue clearing has been a pivotal technique for the latter as it renders tissues transparent, facilitating deep imaging of intact specimens. This method removes the lipids that cause light scattering while preserving the structure and molecular integrity of the tissues, thereby permitting unprecedented visualization of whole complex biological structures using fluorescent microscopy. Popular protocols include CLARITY[6], which involves embedding tissue in a hydrogel and subsequently extracting lipids, and iDISCO[7], which is effective for clearing and staining very large tissue samples such as the whole brain. Whole-organ images of cleared tissue can be taken with a light-sheet microscope, whereby a thin sheet of laser light illuminates a single plane of the specimen at a time. Fluorescence from this plane is then captured perpendicular to the light sheet, allowing for rapid reconstituted imaging of large tissue volumes with reduced light exposure[8].

Regulation of reporter transgene expression strongly influences biological readout

Specific and diverse aspects of biology can be imaged through manipulation of transgene expression. This can be regulated at different levels, which in turn impacts the characteristics of the imaging readout (see Fig. 1a).

At the transcriptional level

The promoter chosen to regulate transcription of the reporter transgene can have a profound influence on imaging readout. Promoters usually range in size from 0.5 to 2 kb and predominantly come in three classes: constitutive, tissue-specific, or conditional.

Constitutive promoters, frequently derived from either viral or eukaryotic "house-keeping" genes such as PGK (phosphoglycerate kinase) or EF1a (elongation factor 1 alpha), can be thought of as being "always on". The intensity of the imaging signal is therefore proportional to labeled cell number, not biological state, and so they are frequently used to non-invasively track the location and longevity of implanted cell populations or

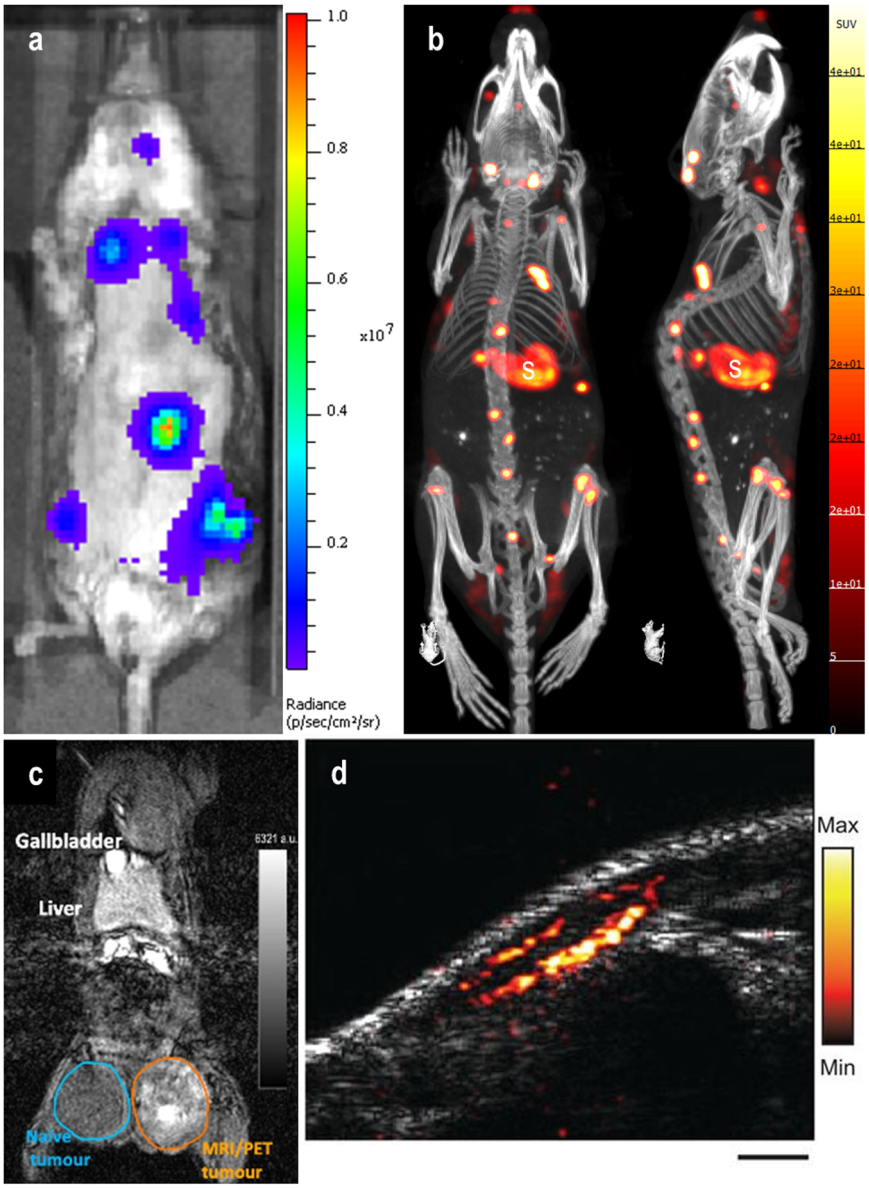

Fig. 2 | Examples of constitutively expressed reporter transgene imaging in preclinical oncology. Firefly luciferase-based bioluminescent imaging (**a**) gives a rapid and sensitive readout of the spread of labeled murine lung adenocarcinoma metastases following the intracardiac injection of 412 P cells 3 weeks earlier. However, BLI signal is surface-weighted and prone to scatter and absorption from overlying tissues. **b** A SPECT/CT image of the same mouse taken three days later and the uptake of [^{99m}Tc]$NaTcO_4$ mediated by mNIS, which is co-expressed with luciferase in these cells. This tomographic image is lower throughput and involves a radioactive tracer, but highlights multiple individual metastatic lesions in bone and soft tissue that are otherwise not evident by BLI. "S" indicates signal from the stomach (an organ that expresses high endogenous levels of NIS) and is not related to tumor development. **c** The enhancement of T1-weighted MRI signal conferred by the expression of organic anion-transporting polypeptide (OATP), which increases the uptake of gadolinium-based MRI contrast agents such as Gd-EOB-DTPA in labeled tumor cells. **d** An ultrasound image of a subcutaneous tumor that is expressing the recently developed mammalian acoustic reporter genes (mARGs). Transfected cells express gas vesicles which can be collapsed by applying a specific ultrasound frequency. Comparing the signal intensity before and after the application of this frequency produces a specific heatmap, proportionate to reporter expression. *(a) and (b) courtesy Spyridon Champeris Tsaniras and Linda Van Aelst, Cold Spring Harbor Laboratory. (c) reprinted with permission*[76]*. (d) reprinted with permission*[74]*.*

to monitor tumor development over time (e.g.[9]; see Fig. 2). Viral promoters (e.g. CMV (cytomegalovirus)) are usually strong and express reporters at a high level, but may be prone to epigenetic silencing in certain cell types such as embryonic stem (ES) cells or organoid cultures.

Tissue specific promoters, originally derived from a specific endogenous gene (e.g. pancreatic-specific Ptf1a (p48) promoter, astrocyte-specific Aldh1l1 (aldehyde dehydrogenase 1 family, member L1) or GLAST (glutamate aspartate transporter) promoters[10–12], and Fig. 3c), can be used to restrict reporter transgene expression to a specific type of cell, even when the reporter transgene cassette is integrated into the genome of cells in many different organs (e.g. in a transgenic mouse). These promoters can either be employed to directly express the reporter, or to drive the expression of Cre or FLP recombinase to specifically activate conditional reporter expression after deletion of a floxed or FRT'd stop cassette (see post-transcriptional section). It should be noted that the fidelity of reporter expression from a specific promoter is not always guaranteed, as not all regulatory elements are necessarily present within the short promoter sequences typically employed. Transgene expression will also be influenced by where in the genome it randomly integrates (the position effect). This issue can be addressed by either "knocking-in" the entire reporter expression cassette to a defined "safe-harbor" locus, such as *ROSA26*[13] (reverse orientation splice acceptor) or Col1A1 (collagen type I alpha 1), or by highly efficient and precise gene targeting of the reporter transgene to specific endogenous loci using CRISPR/Cas9[14,15]. Additionally, copy number (the number of stably-integrated transgenic cassettes) will influence overall expression level and cells with a higher copy number will typically appear brighter on the scanner. A clonal population of single-copy cells will show more predictable imaging performance however and this can be readily achieved by targeting the transgene expression cassette to a specific genomic locus, or with a relatively low multiplicity-of-infection (e.g. MOI of 1 or less) of viral vector relative to plated cell number, followed by selection. Copy number can also be increased, either by increasing viral vector MOI, or by transfecting plasmid DNA, which tends to concatemerize via extrachromosomal recombination prior to random integration in the genome at a single genomic location. Logically, polyclonal cell populations with different transgenic copy numbers will appear brighter, but may exhibit more heterogeneous imaging performance, especially so in studies of tumor metastasis whereby distal tumors form from the outgrowth of a single cell.

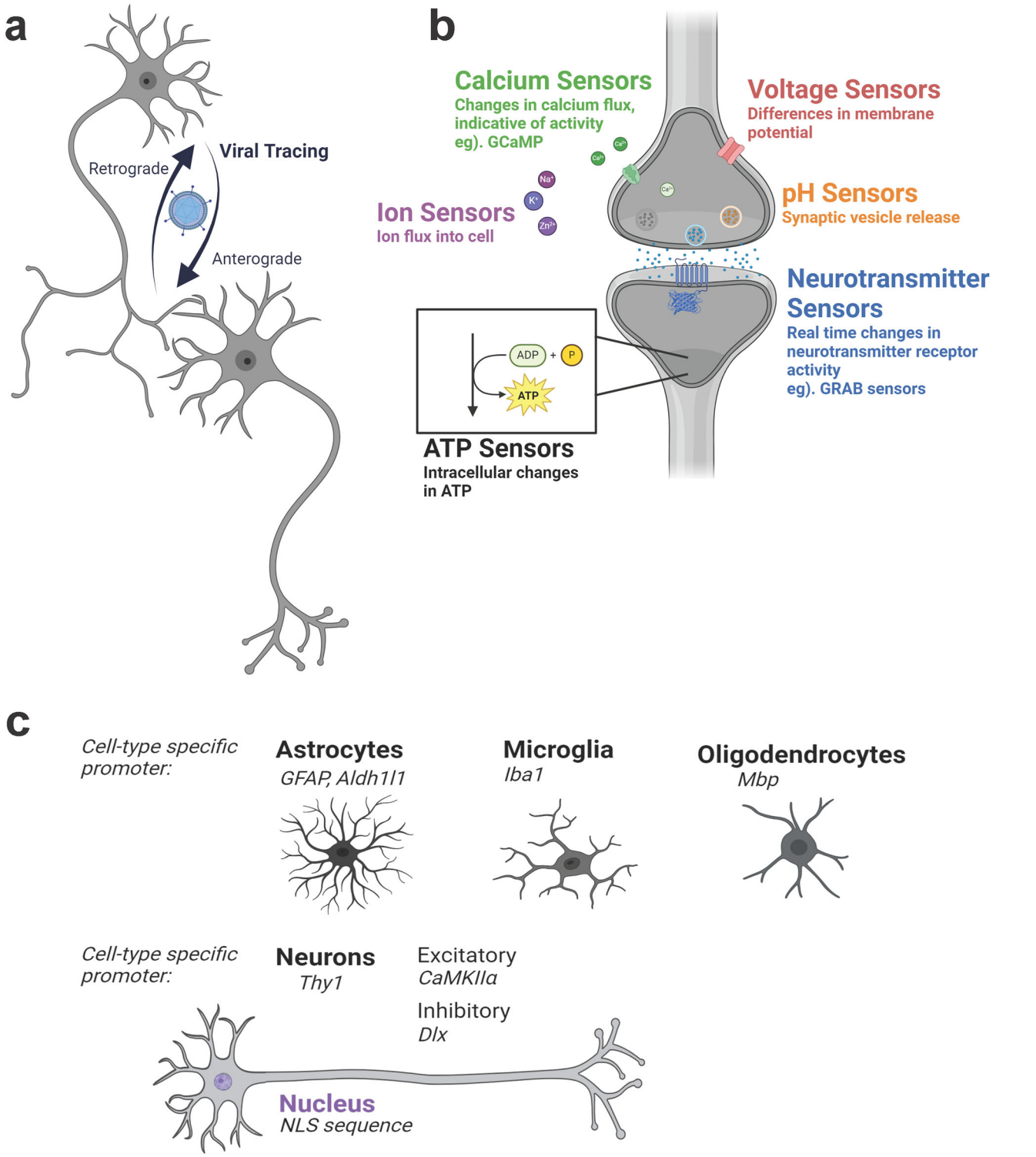

Fig. 3 | Approaches to monitor specific aspects of neuronal and glial cell activity in vivo and in vitro. a Viral tools can be used to transduce neurons in the anterograde (from soma to axon terminal) or retrograde direction (from axon terminal back to soma) to map inputs and outputs of select neural circuits. **b** A whole suite of transgenic tools has been developed to monitor intracellular calcium, potassium, sodium, zinc, ATP, pH, membrane voltage, and neurotransmitter and neuromodulator concentrations. **c** These tools can be targeted to specific neuronal or glial cell populations via the use of different promoter sequences, or to different subcellular compartments or organelles by adding a specific peptide targeting sequence to the transgene (e.g. NLS). (Created in BioRender. Battison, A. (2024) https://BioRender.com/f08h833).

Conditional promoters come in two types; those that are active in the context of a specific biological state (e.g. NF-κB (inflammation[16]), or E2F transcriptional reporter (cell cycle[17])), or those that are active only in the presence of a specific molecule. In that regard, tetracycline-inducible "on" or "off" systems are popular[18], due to relatively low levels of leakiness when off and good fold induction when on. Again, these can be used to either directly drive the expression of a reporter, or combined with a tissue-specific Cre or FLP recombinase transgene and conditional (floxed or FRT'd stop) reporters.

At the post-transcriptional level

Reporter function can also be regulated at the mRNA level prior to translation. For example, a widely employed approach to achieve tissue- or cell-type specific reporter transgene expression in both preclinical cancer and neuroscience research is to employ a floxed (or FRT'd) stop cassette (see Fig. 1a). When positioned between the promoter and reporter transgene coding sequence, translation of the full-length mRNA transcript is effectively halted by one of multiple stop codons present in all three reading frames. The stop cassette can be removed in cells that also express Cre (or FLP) recombinase however, resulting in translation of the reporter protein and imaging signal from recombined cells only (e.g.[19]).

A famous example of an imaging readout driven by Cre recombinase is the "Brainbow" mouse[20]. Originally used to trace individual neurons in the brain, the brainbow (or derivative) allele randomly generates multiple unique colors from a tandem array of three or four fluorescent reporters, each flanked by mutant loxP sites. Transient Cre expression results in the partial and random recombination of the brainbow cassette, which in turn can give rise to the essentially random generation of up to 100 different fluorescent colors, thus enabling the mapping of individual neurons, even when they are in close proximity.

Another example of an imaging readout regulated at this level detects and quantifies ER (endoplasmic reticulum) stress. Xbp1 is an endogenous gene that is alternatively spliced in the context of ER stress. Accordingly, when the 5' sequence of Xbp1 is positioned immediately upstream of a reporter coding sequence in the appropriate reading frame, it too can be rendered ER stress-responsive[21].

Two or more reporter transgenes can be expressed from the same promoter via the construction of a multicistronic construct and this can be an effective way to combine the experimental advantages of different imaging modalities (e.g. the whole-body imaging sensitivity of BLI coupled with single cell microscopic visualization by intravital microscopy). This can be as simple as making a peptide fusion of all reporters by placing their coding sequences in frame relative to each other without internal stop codons[22], but this approach may unintentionally diminish gene function. Alternatively, at the post-transcriptional level, an IRES (internal ribosome entry sequence[23]) or 2A sequence[24] can be positioned between reporter transgene coding sequences. In both instances, a single multigenic mRNA molecule is transcribed. Ribosomes can bind to both 5' and IRES sequences of the mRNA, resulting in the translation of both encoded transgenes as independent proteins. Alternatively, a peptide bond fails to form between the glycine and proline residues in the terminal PGP motif of the 2A sequence. Procession of the ribosome continues along the mRNA however and the downstream protein is translated as a physically separate peptide. It can be challenging to predict the performance of IRES sequences and frequently the upstream gene is expressed to a significantly higher level than the downstream gene. 2A sequences are generally considered to result in the equimolar expression of both reporter proteins, which can be advantageous as it allows the assumption that cells that appear bright by one modality will also appear bright on the other. It should be noted that the upstream gene will retain a peptide tag encoding the majority of the 2A tag at its C terminus (~ 20 amino acids), which may affect functionality in some instances.

At the post-translational level

The function of a reporter transgene can also be regulated at the protein level. This can be an attractive imaging strategy as it can create reporters that respond rapidly to changes in biology. For example, peptide domains called degrons can be fused to the N- or C-terminus of a reporter, leading to differential protein stability during defined biological states. When functionally active, degrons promote the continuous ubiquitination and degradation of the reporter protein. This process is halted in the context of certain biology however, resulting in stabilization of the reporter transgene and rapid induction of imaging signal. This strategy was used to make the FUCCI cell cycle reporter system[25], with the geminin degron conferring reporter stability and green fluorescence during replicative phases of the cell cycle (S, G2, and M phase), whilst the CDT1 degron conferred stability and red fluorescence solely at G1. Similarly, the Nrf2 degron has also been used to generate a rapid imaging readout of oxidative stress[5,26]. In comparison to reporter transgenes regulated at the transcriptional level (whereby signal first relies upon transcription, translation, protein folding, and possibly translocation to a specific sub-cellular location), induced signal from a degron-based transgene is evident rapidly, in less than an hour, as it results from the stabilization of an otherwise constantly expressed but degraded protein.

The functionality of Cre recombinase can also be temporally regulated at this level, with the fusion of an ER^{T2} domain (mutated estrogen-receptor domain)[27]. In the absence of ligand, 4-hydroxytamoxifen (4-OHT), Cre recombinase protein is restricted to the cytoplasm and so it is unable to recombine DNA. Upon the administration of 4-OHT however, Cre protein translocates to the nucleus, where it can functionally recombine DNA at loxP sites.

Synthetic biology approaches termed circular permutation and allostery, which essentially involve the fusion of reporter transgenes with other biologically relevant proteins or peptide domains, have also created many specific imaging biosensors that generate signal in response to biology (e.g. GCaMP as a genetically-encoded sensor for calcium[28] and iGluSnFR as a sensor for glutamate[29]; both discussed later in the "Functional imaging of ions" section). Post-translational regulation of reporter function can also facilitate imaging of specific protein: protein interactions within the cell. Popular fluorescent or bioluminescent reporters have been split into two separate N- and C- terminal fragments, that when co-expressed do not associate or generate signal. Reporter function is partially restored when both fragments come into proximity however, so when fused to interacting peptide domains, reconstituted signal indicates specific binding of those two proteins (e.g.[30]). Similarly, protein interactions can also be imaged via a process called FRET (Förster Resonance Energy Transfer)[31]. Only when in immediate proximity, the signal emitted from one excited fluorescent protein is able to excite emission from the second fluorescent protein. Thus, when both colors are fused to proteins relevant to the assay, red-shifted light only arises with protein: protein interaction and the fluorescent proteins are brought together. A split reporter strategy has also been used to indicate the activation of cellular signaling pathways, whereby both inactive components of the split reporter are fused together by an intervening peptide linker. When the linker is phosphorylated by a specific cellular kinase, the molecule undergoes a conformational shift, bringing together the two nonfunctional reporter domains and generating signal[32]. Similarly, GRASP (GFP reconstitution across synaptic partners)[33] has been used in neuroscience to identify synaptically-connected neurons.

Specific considerations for imaging reporters in Neuroscience

The nervous system is comprised of excitable cells which communicate with one another via neurotransmission. Release of neurotransmitter occurs after an electrical action potential results in calcium channels opening, increasing intracellular calcium levels and triggering vesicles within the neuron, packed with neurotransmitter, to fuse to the plasma membrane and release their contents into the extracellular space of the synaptic cleft. The neurotransmitter then diffuses across the synapse where it binds to the post-synaptic cell and the process repeats. There are therefore numerous levels through which neuronal activity can be functionally measured.

Functional imaging of ions

The unique property of chemical synaptic transmission in neurons has resulted in the development of a wide range of tools for measuring active cells (see Fig. 3). Transgenic approaches for detecting ion changes in neurons have revolutionized our understanding of neuronal activity, particularly through the detection of calcium ions, which are vital for neuronal signaling. One of the most widely used transgenes for calcium detection is GCaMP[28], a genetically encoded calcium indicator (GECI). GCaMP consists of a fusion protein that combines a calcium-binding protein (calmodulin), a peptide sequence (M13 peptide from myosin light chain kinase), and a

fluorescent protein (GFP). When calcium ions bind to calmodulin in GCaMP, a conformational change occurs, altering the fluorescence of GFP. This change in fluorescent intensity is directly proportional to the amount of calcium present, thus allowing real-time quantitative imaging of calcium dynamics in live neurons. This method provides insights into the spatial and temporal patterns of neuronal activity across different conditions and treatments, offering a powerful tool for neuroscience research that probes how neurons communicate and respond to a variety of stimuli across millisecond, second, and minute timescales. In addition to calcium, several other ions crucial for neuronal function can be detected using genetically encoded indicators similar to GCaMP. For instance, FRET-based sensors like GEPIIs (genetically encoded potassium ion indicators) have been developed for real-time monitoring of intracellular potassium levels, an ion essential for maintaining the resting membrane potential and action potential propagation in neurons. These tools can be selectively expressed in specific sub-cellular compartments (e.g., cell membrane[34], dendrites, soma, or axons[35]) to examine how ionic flux changes in different parts of the cell. For example, SomaGCaMP7f[36], which selectively targets GCaMP7f to the neuronal soma, was created by fusing the C-terminus region of the human AnkyrinG protein with the ER2 trafficking sequence from the Kir2.1 potassium channel. Additionally, a de novo designed coiled-coil peptide, EE-RR, fused to the C terminus of GCaMP via an amino acid linker promotes selective expression of functional GCaMP7f in neuronal soma.

Imaging neurotransmitters

Neurotransmitters can also be imaged with reporter transgenes, providing spatial and temporal insights into neurotransmitter release and reception at synapses. For example, sensors like iGluSnFR[29] have been developed for glutamate, the most abundant excitatory neurotransmitter in the brain. This sensor fluoresces upon binding to glutamate, allowing researchers to track its release and clearance during neuronal communication. Similarly, dLight1 targets dopamine, a key neurotransmitter involved in reward, motivation, and motor control, offering a window into the rapid dynamics of dopaminergic signaling. Indeed, a whole suite of neurotransmitter and neuropeptide reporters has been developed by systematically engineering specific GPCRs (G-protein coupled receptors) as the ligand-sensing unit and a circularly permuted green fluorescent protein (cpGFP) as the reporter. The sensitivity and reporter dynamics can be modified by adjusting the cpGFP placement site within the GPCR third intracellular loop (ICL3), allowing detection of specific neurotransmitters and neuropeptides with nanomolar affinity[37]. Generated in a similar fashion, functional imaging has also been expanded to include sensors for molecules like ATP (e.g. GRAB-ATP, MaLion) and pH (pHluorin), allowing real-time monitoring of factors that are critical for cellular metabolism and homeostasis.

Voltage sensors

Voltage sensors are innovative tools in neuroscience, designed to directly monitor the electrical activity of neurons by detecting changes in membrane potential, which directly relates to neuron activity and the action potential. These sensors often operate based on conformational changes in their structure that occur in response to voltage fluctuations across the cell membrane. This change alters their fluorescent properties, enabling real-time visualization of action potentials and subthreshold voltage changes within neurons[38]. For instance, ASAP and JEDI-2P are voltage sensors derived from cpGFP which provide fast and sensitive responses to voltage changes. These sensors have proven to be especially useful for tracking rapid neuronal activity[39,40].

Reporter transgene labeling approaches also influence biological readout

The approach taken to introduce reporter transgene expression can also significantly influence subsequent in vivo imaging outcomes. In the case of implantable models, whereby the target cell population is first propagated in vitro before in vivo implantation and imaging, there are many experimental options available to the researcher.

In preclinical cancer research, as tumor cells are hyperproliferative, a delivery approach that results in the stable integration of the transgene into the recipient cell genome will frequently be employed (see Fig. 1d). Transgenic DNA will then be replicated along with the recipient cell genome at each cell division so, on condition that the reporter is not causing a selective disadvantage and so prone to deletion or epigenetic silencing, expression will be retained in successive generations of daughter cells, enabling imaging over the course of tumor development.

Nonviral or viral vectors can be employed to achieve this. In vitro, a nonviral approach could be as simple as the transfection of a plasmid with a lipid or net positively charged reagent (e.g. lipofectamine or PEI (polyethylene imine)), then selecting for stable transfectants using an antibiotic. The percentage of cells with stably integrated transgenic DNA can be greatly increased after transfection with the use of a transposon system (e.g. sleeping-beauty or piggyBac), which is sufficiently efficient to even achieve genomic integration in vivo[41,42]. As nonviral vectors are plasmid-based, they are not bound by the same packaging limits as viral vectors and, depending on their cargo, should be less immunogenic, especially so their minicircle derivatives[43]. Due to a lack of intrinsic infectivity, in vivo transgenesis with nonviral vectors can be challenging, although protocols have been developed to efficiently target specific organs. For example, cells in the lung can be targeted via inhalation of vector plus transfection reagent[44], or cells in the liver can be genetically modified via hydrodynamic tail vein injection, whereby plasmid DNA in a relatively large volume of PBS (phosphate buffered saline) is injected rapidly via the tail vein[45]. Nonviral vectors can also be delivered to specific organs via a process called sonoporation. First, a mix of the DNA vector with microbubbles (an ultrasound contrast agent) is either injected intravenously or directly into the organ of interest. High-frequency ultrasound is then administered to the region of interest to burst the bubbles, resulting in DNA uptake through transiently compromised cell membranes[46]. Electroporation is another widely used technique to introduce transgenic DNA into cells, both in vitro and in vivo, whereby a brief electric field is applied to temporarily disrupt the cellular membrane, allowing DNA or other molecules in proximity to enter (see Fig. 4b)[47].

Viral vectors

Attenuated and replication-incompetent viral vectors are popular experimental tools to introduce reporter transgene expression (see Table 2). Although these vectors first require a packaging step, a significant advantage over nonviral delivery methods is that they are intrinsically infective at least once and can greatly facilitate gene delivery to a broad number of cell types in vitro or in vivo, with little need to optimize experimental conditions. Their intrinsic infectivity, especially of viral vectors infective to human cells (amphotropic), necessitates handling at a higher level of biosafety than nonviral vectors however.

Replication incompetent and VSV-G (vesicular stomatitis virus glycoprotein) pseudotyped lentiviral vectors are popular in both preclinical oncology and neuroscience research. They are broadly infective to most mammalian cell types and can package up to around 7 kb of DNA, which is a useful amount for many transgenic expression strategies. For biosafety purposes, researchers commonly employ 2nd or 3rd generation systems to package lentiviral vectors[48]. These systems increasingly separate the essential viral packaging genes (GAG/POL, ENV and REV) needed to package a pseudotyped-lentiviral particle, greatly reducing the chance that random recombination events produce a replication-competent vector. Importantly, lentiviral vectors deliver stable transgene expression to nondividing cells, either growing in vitro or to somatic cells in vivo. The efficiency of gene delivery with viral vectors is proportional to the number of infectious units administered. Frequently, this key experimental advantage supersedes the effort needed to package and titer viral stocks prior to transgene delivery.

Other viral vectors commonly employed in preclinical oncology research include replication-incompetent adenovirus (AV), which can be easily grown to high titers in vitro in a packaging cell line. AV does not integrate into the genome of transduced cells, but rather is maintained as an

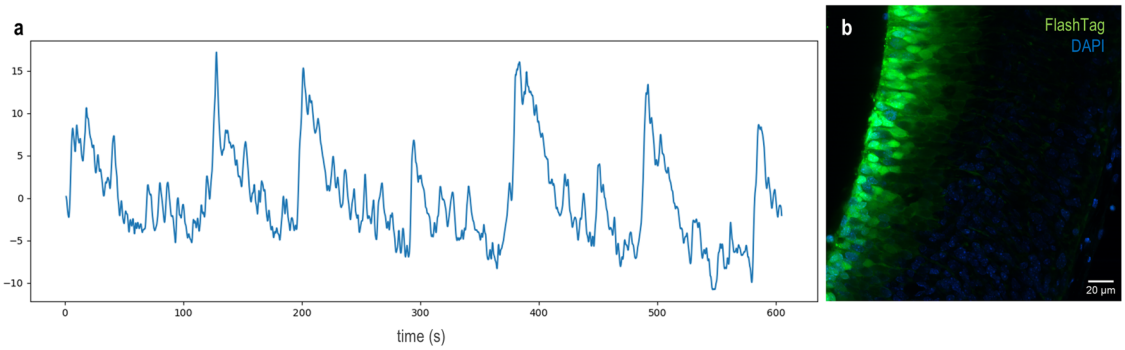

Fig. 4 | Example of structural and functional imaging in the brain. A ten-minute GCaMP6s trace in vesicular GABA transporter- (VGAT-) expressing neurons in the suprachiasmatic nucleus of a freely moving mouse (**a**). The amplitude of the signal is directly correlated with calcium ion concentration and neuronal activity. The multiphoton microscope image in (**b**) shows the ventricular wall of a mouse embryo at E15. Mice were electroporated in utero with DAPI (blue) and FlashTag (green), a process that labels M phase neural progenitor cells across the CNS with carboxyfluorescein esters. Replicating radial glial cells are therefore selectively highlighted by FlashTag in the fluorescent image. *(a) courtesy Adrian Gomez, Cold Spring Harbor Laboratory. (b) courtesy Debby Silver, Duke University and Kel Sakaki, Scientifica Ltd.*

Table 2 | Popular viral vectors for imaging in cancer and neuroscience research

Virus	Primary Application	Approximate Packaging Limit	Stable Integrant in the Genome	Notable Features
Lentivirus	Cancer and neuroscience	7 kb	Yes	VSV-G pseudotyped virus is broadly infective. Suitable for long-term transgene expression, reasonable packaging limit
Adenovirus	Cancer and neuroscience	8 kb	No	Produces to high titers in engineered packaging cells, delivers transient transgene expression
AAV (adeno-associated virus)	Cancer and neuroscience	4.5 kb	Potentially yes	Versatile as vector tropism is serotype-dependent
Pseudo-rabies virus	Neuroscience	8–10 kb	No	Travels polysynaptically in the retrograde direction
Yellow fever virus	Neuroscience	11 kb	No	Good packaging size compared with AAV, but high immunogenicity limits certain applications
CAV-2 (canine adenovirus)	Neuroscience	~30 kb	No	Preferential tropism for neurons, large packaging size and low immunogenicity
HSV (herpes simplex virus)	Neuroscience	30–50 kb	No	Large packaging size and ability to trace nerves in the anterograde direction

episome and so is gradually lost over successive rounds of cell division. For imaging, adenoviral vectors are frequently used to deliver the transient expression of Cre recombinase to permanently switch on or off the expression of floxed model-relevant alleles or reporters.

In neuroscience, retrograde and anterograde tracing are essential techniques used to elucidate the architecture and connectivity of neural circuits (see Fig. 3a). Retrograde tracing is used to identify the neurons that project their axons to a specific target area. In this method, tracers or labeling substances are taken up by the axon terminals and transported back to the cell bodies, revealing the origins of the input to the targeted region. Common retrograde tracers include fluorogold, cholera toxin subunit B, and pseudorabies virus (PRV). Conversely, anterograde tracing maps the destinations of axons emanating from a specific group of neurons, and includes some AAV (adeno-associated virus) serotypes, herpes simplex virus (HSV) and rhodamine isothiocyanate (RITC). Both tracing techniques are pivotal in providing a directional map of neural pathways, helping scientists understand how different parts of the brain communicate and process information.

Pseudorabies virus (PRV) is a member of the Herpesviridae family and is commonly employed to trace neurons via their synaptic connections[49]. Despite its name, it is not related to the rabies virus but does share the characteristic retrograde trans-synaptic movement of that virus. As a result, PRV is employed as a powerful tracing tool to map neuronal circuits, as it allows for backtracking from injection location through the neural circuitry. It can be genetically modified to express fluorescent proteins or other markers, enabling researchers to visualize neural connections across different brain regions, or between the brain and body. This makes PRV an invaluable tool for studying the complex architecture and function of neural networks.

AAV tropism refers to the intrinsic ability of different serotypes to infect specific cell types or tissues[50]. This selectivity is largely governed by the viral capsid structure, which determines the AAV's ability to bind to cellular receptors and transduce particular cells. There are numerous serotypes of AAV, each exhibiting distinct tropism characteristics. For instance, AAV serotype 1 (AAV1) effectively transduces muscle cells, while AAV serotype 2 (AAV2) has a natural tropism for neurons and retinal cells. These tropisms can be exploited to deliver genetic material specifically to desired cell populations, enhancing both the efficiency and specificity of transgene delivery[51,52]. While AAVs are often injected locally, directed evolution has resulted in specific AAV serotypes which infect either the whole brain (AAV.PHP.B and AAV.PHP.eB) or the periphery (AAV.PHP.S) with a simple intravenous injection[53,54].

Germline approaches

Reporter transgene expression can also be introduced into a transgenic model of interest through the germline[55]. Once reporter transgene mice have been generated and validated, they can be bred with other transgenic models to render them imageable. Depending upon the complexity

of the model and the number of alleles that need to be combined, at around 3 months per cross, this can be a time-consuming process and experimental success cannot be assessed until all alleles have been combined. The recent development of CRISPR/Cas however, stands to revolutionize our ability to generate accurate imaging reporter transgenes in both preclinical cancer and neuroscience research. Rather than relying on just a short promoter fragment to achieve reliable expression (as depicted in Fig. 1), transgenes may be efficiently inserted into a precise locus in the genome, such that all the regulatory elements that influence transcription of the endogenous allele also influence the reporter. Traditionally, targeting a transgene to a specific location in the genome via homologous recombination was inefficient and laborious. Following the demonstration that linear DNA can efficiently be used to repair the double-stranded DNA break induced by Cas9, with or without homologous sequence[14,56], CRISPR has made it feasible to generate precisely targeted reporter transgenes almost at will in a wide variety of cell types, not just murine ES cells[57].

To reduce the complexity of combining multiple inherited alleles through breeding, a hybrid approach, partly reliant on breeding, partly on somatic gene transfer, has proven useful in the context of rendering highly penetrant transgenic cancer models imageable. For example, lentiviral vectors have been developed to deliver Cre recombinase expression (to induce tumors) along with stable reporter transgene expression. In the context of the LSL-Kras G12D, p53 fl/fl mouse, bioluminescent NSCLC can be induced following inhalation of a Cre and Fluc vector[58]. Similarly, imageable prostate tumors or sarcomas can be induced following the appropriate injection of a similar lentiviral vector in p53 fl/fl, PTEN fl/fl mice[59,60].

It should be noted that the somatic introduction of nonendogenous reporter transgenes such as GFP or firefly luciferase may be immunogenic when expressed in the context of an immune-competent mouse. This is especially pertinent with cell implantation models and immunotherapy research, even if the implanted cells are syngeneic (genetically matched) with the recipient. Several studies have shown that immunogenicity from reporter transgene expression can affect tumor biology, slowing primary tumor growth and preventing metastatic spread[61]. To counter those effects, researchers have developed transgenic mice that express low levels of reporter at body locations distal to the region of interest and so effectively immune-tolerize the mouse to subsequent reporter-expressing cell implants[62,63]. Another strategy has been to develop a vector, that via a two-step labeling approach, enables efficient cell labeling with only mNIS expression (murine sodium iodide symporter) for PET or SPECT imaging. As an endogenous gene, mNIS is not immunogenic in such an experimental context[64]. For similar reasons of immune compatibility, the human isoform of this gene has attracted significant attention in cell tracking[65] and oncolytic virus research in the clinical setting.

Conclusions

Two subject areas that have benefitted tremendously from the development of biological imaging tools are neuroscience and cancer biology. In recent years, researchers have begun to integrate approaches and concepts from these two fields under the common umbrella of "cancer neuroscience"[66,67]. This stems from the growing appreciation that nerves are a critical component of the tumor microenvironment (TME), and that tumor-innervating nerves communicate with multiple cell-types within the TME to drive tumor progression and metastatic spread. We discuss here how the expression and delivery of different reporter transgenes for different imaging modalities can be modified to achieve diverse biological readouts. A major benefit of combining tools from neuroscience and cancer research is that they often provide orthogonal information. For example, monitoring tumor innervating nerve activity using germline or viral expression of GCaMP8s in combination with tumor cells that constitutively express a luciferase would allow researchers to visualize both nerve activity and developing tumor burden in living mice simultaneously. Alternatively, using optogenetics to manipulate tumor-innervating nerves in tandem with different reporters within the TME (e.g., hypoxia or reactive oxygen species (ROS)) would allow for researchers to causally relate neuronal activity with relevant physiological changes directly in the tumor. We are only just beginning to integrate the wealth of tools across cancer biology and neuroscience. By modifying transgene structure, expression, tissue/cell-type specificity, cellular localization, or subsequent degradation, we can best address critical open questions at the intersection of these two fields.

Data availability

No datasets were generated or analysed during the current study.

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Competing interests

The authors declare no competing interests.

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