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Transgenic mice encoding modern imaging probes: Properties and applications

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SUMMARY

Modern biology is increasingly reliant on optical technologies, including visualization and longitudinal monitoring of cellular processes. The major limitation here is the availability of animal models to track the molecules and cells in their natural environment *in vivo*. Owing to the integrity of the studied tissue and the high stability of transgene expression throughout life, transgenic mice encoding fluorescent proteins and biosensors represent unique tools for *in vivo* studies in norm and pathology. We review the strategies for targeting probe expression in specific tissues, cell subtypes, or cellular compartments. We describe the application of transgenic mice expressing fluorescent proteins for tracking protein expression patterns, apoptotic events, tissue differentiation and regeneration, neurogenesis, tumorigenesis, and cell fate mapping. We overview the possibilities of functional imaging of secondary messengers, neurotransmitters, and ion fluxes. Finally, we provide the rationale and perspectives for the use of transgenic imaging probes in translational research and drug discovery.

INTRODUCTION

Optical imaging, readout, and sensing have become integral approaches in modern biomedical research. Transgenic mice expressing fluorescent proteins (FPs) and optical biosensors have a range of advantages for application in basic and translational studies. Genetically encoded FPs and biosensors (Shcherbakova et al., 2012; Shcherbakova and Verkhusha, 2014; Leopold et al., 2019) provide a wide range of possibilities for the *in vivo* analysis of disease etiology and pathogenesis and enable relatively rapid large-scale screening of the drug efficiency and toxicity in preclinical studies.

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AUTHOR CONTRIBUTIONS

V.V.V. conceived the project. L.A.K. and V.V.V. wrote the manuscript. L.A.K. made figures and tables. Both authors reviewed and revised the manuscript.

SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.

In vivo validation is now an essential step in the majority of studies of biological processes. *In vitro* experiments do not consider tissue heterogeneity and integrity with complex hormonal regulation and homeostatic response, accomplished in the whole organism. At the same time, all the above mentioned have a robust impact on tissue metabolism, differentiation, regenerative, and tumorigenic potential. For some organs, like a brain, any tissue disruption greatly simplifies the sort of experimental data obtained and still questions the implementation of obtained knowledge to intact processes (Gonzalez-Riano et al., 2021). It is not uncommon for *in vitro* data on neurotransmission to be opposite to what later emerged from studies on the intact brain. For some types of signaling, like the endocannabinoid system, tissue disruption itself rapidly activates or impairs endogenous cascades, affecting ligand production and making their actual parameters and fluctuation inaccessible (Kasatkina et al., 2021). Owing to these challenges, the development of genetically encoded probes has usually been followed by the generation of transgenic mice expressing imaging reporters and drives the evolution of imaging techniques with minimally invasive intervention and/or high spatiotemporal resolution.

Current approaches for *in vivo* visualization include positron emission tomography, magnetic resonance imaging (MRI), single-photon emission computed tomography, and optical fluorescence and bioluminescence imaging. By reviewing the opportunities provided by fluorescent reporters, we refer those readers interested in other imaging modalities to other specific reviews covering such topics. Here, we characterize available and prospective mouse models encoding imaging reporters of visible-light spectrum, including recently developed near-infrared (NIR) FPs and biosensors (Shcherbakova et al., 2018; Matlashov et al., 2020; Oliinyk et al., 2019; Hochbaum et al., 2014; Adam et al., 2019; Shemetov et al., 2021). While visible light has low tissue penetration and high scattering and interferes with body autofluorescence, NIR light overcomes these limitations. Thus, NIR imaging reporters provide an opportunity for non-invasive or minimally invasive applications to monitor cell dynamics and cellular metabolism in intact tissues at a greater depth compared with commonly used green fluorescent reporters. Mouse strains encoding NIR FPs and optogenetic tools acting in the NIR light spectrum are available for non-invasive imaging, transcription regulation, protein targeting, and enzymatic activity, among others (Tran et al., 2014; Fukuda et al., 2019; Hock et al., 2017; Kulathunga et al., 2018; Kasatkina et al., 2022).

Tissue heterogeneity still requires the development of genetic tools with narrow specificity for precise labeling, recording, or optogenetic manipulations of cellular populations. Simultaneous development of multiple Cre-driver strains expanded the potential for targeting imaging reporters and long-term *in vivo* studies of multiple cellular processes. Among them, mouse models of human diseases significantly facilitate the screening of new drug and vaccine candidates to evaluate the toxicity, pharmacokinetics, and pharmacodynamics in preclinical studies.

Conditional and inducible optical probe expression in organs, tissues, and cells *in vivo* conferred new opportunities for tracing individual cell populations during embryogenesis, tissue differentiation and regeneration, and malignant transformation. Multiplexing of fluorescent reporters in transgenic mice largely advanced morphological and functional

brain mapping. Precise targeting of a specific population of cells and even cellular compartments is now possible with localization signals, protein fusions, and the use of promoters with narrow specificity. In line with morphological tracing enabled by a wide range of cell-specific promoters, functional discrimination among highly heterogeneous cell populations can be achieved with activity-dependent promoters.

In this review, we analyzed the strategies for the spatiotemporal targeting of genomically encoded imaging reporters and discuss their advantages for application in biomedical studies. We describe strategies for the generation of new or the selection of available transgenic mouse models for the long-term tracking of organelles, cells, or tissues, which are essential tasks in basic research, including cell and developmental biology, stem cell biology, neuroscience, immunology, and translational studies, in particular oncology, regenerative biology, and neurodegeneration. We discuss choices of imaging reporters and their temporal and spatial targeting to locate, characterize, or manipulate intracellular targets or cellular populations. Specific focus is made on *in vivo* applications where animal models have paramount advantages.

SPATIOTEMPORAL TARGETING OF FLUORESCENT PROBES

Tissue-specific targeting and temporal probe expression rely on specific localization signals incorporated during probe design and consider an appropriate combination of the reporters (in case of spectral multiplexing) to enable sufficient resolution during the tracking of cell- or tissue-specific events (Figure 1). An optimized transgene construct is then introduced either using retroviral vector, pronuclear microinjection or transfected embryonic stem cells to obtain founder mice with stable germline transmission. For conditional transgene induction, both tissue-specific promoters and Cre/*loxP* or flippase/P1-flippase recognition target (FRT) recombination systems (Rodriguez et al., 2000) are used. The latter systems not only enable cell-specific expression of the reporter, but can simultaneously introduce the knockout of specific floxed endogenous genes in the targeted tissue. This approach allows studying specific gene functions in distinct cell populations and at the desired stage of development or disease progression.

Moreover, a large set of cellular populations remained non-targetable until the development of new platforms for transgenic animals like TIGRE (for tightly regulated), which increased reporter expression to the levels observed with adenovirus-associated (AAV)-mediated gene delivery (Daigle et al., 2018). This approach is now allowing scientists to express FPs at substantially higher levels than before and to trace individual neurons having thin spines and sending projections to a distant region of the brain (Reardon, 2017; Peng et al., 2021) for the mapping higher cognitive functions. For the studies of the neuronal circuit involved in the specific behavior, spatial and associative memory reporters directed by the activity-dependent promoters of immediate-early genes are used, such as FOS, ARC, and EGR1. They have low basal expression and strong experience-based induction to map brain activity. Bellow, we discuss the general principles of transgene design for conditional reporter expression (Figure 2) and its targeting to subcellular compartments.

Spatial control of reporter expression

A set of cell-type-specific promoters is used to restrict the reporter expression and enhance imaging contrast and resolution of optogenetic manipulation (Table S1). In line with this, separate groups of promoters permit targeting of highly heterogeneous cells, like neurons, discriminate between glutamatergic and GABAergic neurons, cell subtype, region-specific expression, and the ability to produce or respond to certain neuromediators and hormones. The type of vesicular neurotransmitter transporter allows discrimination between glutamatergic and GABAergic neurons. For this purpose, the promoters of vesicular glutamate transporters (Slc17a6/VGlut2 and Slc17a8/VGlut3) or vesicular GABA transporter (Slc32a1/VGAT) are used for targeting. Various promoters can be used for pan-excitatory neurons, such as CALB1, HTRLaa, NPR3, PLXND1, RASGRF2, and RORB (RAR-related orphan receptor β) promoters.

Cre recombinase encoded under specific promoters and delivered by AAV or Cre-driver mouse is an efficient tool for cell-specific expression of floxed imaging reporters. An array of mouse strains, in which excitatory and inhibitory interneurons were labeled using either VGlut2^{Cre} or VGAT^{Cre} and the expression was directed to the spinal cord dorsal horn and barrel cortex using RORβ promoter (Koch et al., 2017), demonstrated that the low-threshold mechanoreceptor recipient zone of the dorsal horn plays a principal role in the somatosensory processing and patterns the activity of ascending touch pathways that underlie tactile perception (Abraira et al., 2017). While selecting a Cre driver mouse (Figure 1), one may note that in some strains Cre may be expressed in the germline or early embryo, which may be undesirable or intentionally used for global recombination. This phenomenon explains why recombination in progeny may occur independent of the inheritance of Cre.

Certain promoters can localize the expression of the reporter to a specific brain region. The promoter of FEZ family zinc finger 1 restricts the expression to the ventromedial hypothalamus, while the promoter of guanine nucleotide-binding protein subunit β-4 targets the expression to the claustrum.

Pro-opiomelanocortin-alpha (POMC)-expressing neurons in the arcuate nucleus of the hypothalamus, in the pituitary gland, and in the neurogenic niches of the dentate gyrus can be labeled with fluorescent reporters encoded under the POMC promoter/enhancer regions. Such *POMC-EGFP* mice may be useful in studying hypothalamic neuronal signaling pathways involved in the regulation of glucose homeostasis, leptin activity, feeding behavior, obesity, and depression. Studies using *POMC-EGFP* mice demonstrated that lactate plays a role of an intercellular messenger in astrocyte-neuron intercellular communication and the regulation of food intake (Ordenes et al., 2021).

Temporal control of reporter expression

In line with the ability to target certain cell populations, researchers can switch on an expression when needed for specific research tasks. Drug-inducible expression of a reporter and Cre/Flp can be achieved with either a drug-inducible promoter or by fusing a recombinase with the protein sensitive to external chemical signals (Figures 2C and 2D). Moreover, reporters and recombinases can be light activated. These approaches allow

switching on the expression of optogenetic tools or imaging reporters at a distinct stage of development or disease and, in some cases, achieving reversible and quantitative control of expression.

In the Tet-On/Tet-Off system, a tetracycline transactivator (tTA) protein, which is a fusion of bacterial tetracycline repressor and the activation domain of herpes simplex virus VP16, binds to TetO sequence and induces expression of a downstream gene. Two types of this system exist with tTA binding operator only in the presence (Tet-On) or absence (Tet-Off) of tetracycline or one of its derivatives, such as doxycycline. Notably, the first mouse strain become available with a temporally controlled expression of Cas9 after doxycycline administration (Bowling et al., 2020). In the Tet-Off system, regulation relies on a tetracycline response element composed of several repeats of the TetO sequence and can be used to design tetracyclin-independent optogenetic tools for *in vivo* gene transcription regulation with Cre and short hairpin RNAs.

Several types of inducible Cre and mammalian-optimized Flp (Flpo) recombinases were generated and available in transgenic mice, providing additional opportunities to control reporter expression *in vivo*. They are encoded as fusions with mutant mouse estrogen receptor G525R, which does not bind its natural ligand 17β-estradiol at physiological concentrations, but is activated by synthetic ligands 4-hydroxytamoxifen and ICI 182780. This Cre-ERT fusion protein is sequestered in the cytoplasm and translocates to the nucleus only upon the binding of tamoxifen. Cre and Flp drivers with improved tamoxifen-inducible CreERT2 and FlpoERT2 fusions demonstrate better induction kinetics, which is one of the critical parameters (Figure 1) for studies of rapid developmental changes in early stage embryos.

Separate chemogenetic approaches are based on inducible recombinases encoded as fusions with a dihydrofolate reductase destabilizing domain (Sando et al., 2013) that mediates their proteasomal degradation, resulting in little or no recombinase activity (Figure 2D). The proteasomal degradation can be blocked by trimethoprim (TMP), which can be easily administered to mice for *in vivo* application. Several transgenic mice encoding TMP-inducible Cre and Flpo were generated for labeling of neuronal subpopulations (Daigle et al., 2018; Tasic et al., 2016) and helped to avoid the interference of tamoxifen stimulation with the studied processes. However, the performance of these types of recombinases is rate limited by the proteasomal machinery, and some background activity may be observed (Sando et al., 2013).

Optogenetic manipulation is also used for spatiotemporal control of reporter expression. For example, temporal control of floxed reporter *in vivo* is possible with light-inducible (photoactivatable) Cre recombinase (Kawano et al., 2016), which uses magnets optogenetic system engineered from the *Neurospora crassa* vivid photoreceptor (Kawano et al., 2016). This photoactivatable Cre is encoded as two separate split Cre segments, such as nMag-CreN59 and pMag-CreC60, and activated with blue light causing the nMag-pMag dimerization (Figure 2D). High efficiency of photoactivatable Cre was demonstrated in primary neural cells and embryonic fibroblasts, as well as in the mouse liver and AAV-transduced mouse brain *in vivo* (Morikawa et al., 2020). A light-inducible Flp recombinase,

although yet not encoded in transgenic mice, can be delivered with AAV for genetic manipulation in mice with an FRT-flanked reporter.

Subcellular targeting

The necessity to localize imaging reporters in a certain compartment of the cell is frequently dictated by the process studied or is required for efficient signal readout. For this purpose, localization and export signals are introduced to sequester reporter expression in a certain compartment of the cell (Figure 3). Subcellular targeting enhances the spatial resolution of optogenetic manipulation, provides actual dynamics of second messengers (i.e., cytosolic, endoplasmic reticulum or nuclear Ca^{2+}, and allows studying intracellular membrane trafficking. The examples include targeting calcium sensors to the endoplasmic reticulum, a fusion of Dendra2 green-to-red photoconvertible monomeric FP (Gurskaya et al., 2006) with the mitochondrial targeting signal to monitor mitochondrial fusion and transport, targeting fluorescent reporter to the plasma membrane for imaging of cell morphology and membrane dynamics (lipid-modified, glycosyl-phosphatidylinositol-tagged FPs), fusions with synaptic vesicle-associated proteins to restrict the reporter to presynaptic active zones.

The first reported nuclear localization signal (NLS) was a seven amino acid sequence PKKKRKV present in a large T-antigen of simian vacuolating virus 40 (SV40) (Kalderon et al., 1984; Lanford and Butel, 1984). The SV40 NLS, nucleoplasmin NLS (AVKRPAATKKAGQAKKKKLD) (Dingwall et al., 1988; Robbins et al., 1991) and, c-Myc NLS (PAAKRVKLD) (Dang and Lee, 1988) share one (monopartite) or two (bipartite) essential clusters of basic amino acids. NLS sequences derived from EGL-13 transcription factor (MSRRRKANPTKLSENAKKLAKEVEN) (Hanna-Rose and Han, 1999) and TUS-protein (KLKIKRPVK) (Kaczmarczyk et al., 2010) are also used for nuclear targeting of reporters in transgenic mice. NLS can be located at either the N- or C-terminus of the reporter. These sequences are the most frequent NLSs used to localize imaging reporters to the nucleus in transgenic mice. However, likely the other NLS variants (Lu et al., 2021) can be also efficient for *in vivo* nucleus targeting. Fusions with histone H2B can be engineered for nuclear reporter targeting (Kanda et al., 1998).

For reporter sequestration in the cytoplasm, a nuclear export signal of cyclic AMP (cAMP)-dependent protein kinase inhibitor α (NELALKLAGLDINKT) (Wen et al., 1995) fused to a reporter is sufficient for rapid nuclear export. Reporters targeted to the endoplasmic reticulum are generated using calreticulin targeting sequence (MLLPVLLLGLLGAAAD) (Fliegel et al., 1989) and retention signal sequence KDEL (Kendall et al., 1992). Mitochondrial targeting of reporters requires an N-terminal localization sequence of cytochrome *c* oxidase subunit VIII (MSVLTPLLLRGLTGSARRLPVPRAKIHSLGDP) (Rizzuto et al., 1989). Optical probes in transgenic mice can be targeted to autophagosomes by fusing with MAP1LC3A protein (Kabeya et al., 2003), which is a specific marker of these organelles.

Several trafficking motifs of the inwardly rectifying potassium channel Kir2.1 are used in optogenetic tools and imaging probes to enhance their targeting to the plasma membrane. C-terminal endoplasmic reticulum export signal (FCYENE) (Ma et al., 2001), N-terminal (RSRFVKKDGHCNVQFINV) (Stockklausner and Klocker, 2003) and C-terminal

(KSRITSEGEYIPLDQIDINV) (Hofherr et al., 2005) signals of the Kir2.1 improve endoplasmic reticulum and Golgi exports of other plasma membrane proteins (Stockklausner and Klocker, 2003; Hofherr et al., 2005; Ma and Jan, 2002; Ma et al., 2002). Localization signals can be introduced at the N and C terminus of the reporter or between the reporters (Gradinaru et al., 2010). Trafficking signals can prevent aggregation of imaging probes in endoplasmic reticulum and improve their localization. The addition of the export signal FCYENE to the C terminus can rescue the trafficking of the reporter from the endoplasmic reticulum and increase its surface localization (Gradinaru et al., 2010). Targeting a reporter to the plasma membrane can be also achieved with an N-terminal anchoring signal (MGCCFSKT) of myristoylated alanine-rich protein kinase C substrate (Muzumdar et al., 2007).

In neurons, a reporter can be targeted to dendrites with a C-terminal sequence of 5-HT2A receptor (Xia et al., 2003) or to cell soma and axons with a Kv2.1 channel C-terminal sequence (Jensen et al., 2017; Lim et al., 2000). Reporters can be targeted to synaptic vesicles when fused with specific protein markers of these compartments, including synaptotagmin 1, synaptophysin, and vesicle-associated membrane protein 2 (Pennuto et al., 2003).

TRANSGENIC MODELS ENCODING FLUORESCENT PROBES

Multicolor FPs

A range of fluorescent imaging reporters was evolved from FPs and are used either as untagged proteins or as protein fusions for volumetric cell and tissue imaging, functional labeling, and multiplexing (Shcherbakova et al., 2012, 2018; Shcherbakova and Verkhusha, 2014). These reporters or their combinations are now widely used in transgenic mice. The most interesting examples of applications of mice with genomically encoded FPs are listed in Table S2.

A separate set of mice that are now drawing specific attention are strains expressing red FPs and NIR proteins, which operate in a tissue transparency window, and are, thus, preferable for minimally or non-invasive imaging. These animal models are used for enhanced resolution imaging of the first stages of development (Gu et al., 2018; McDole et al., 2018), tissue differentiation, regeneration, metastatic events, tissue tropisms exhibited by different viral serotypes, atherosclerotic plaque progression, phagosomes dynamics, and endogenous patterns of protein expression.

There is a long process of engineering the new FP, followed by its thorough characterization before a new transgenic mouse appears. Constitutive (Tran et al., 2014) and inducible (Hock et al., 2017) *iRFP713* mouse models were generated based on the first bright fluorescent NIR FP, termed iRFP713, developed from bacterial phytochrome (Filonov et al., 2011). NIR FPs and optogenetic tools based on bacterial phytochromes require biliverdin IXa tetrapyrrole as a chromophore and fully rely on endogenous biliverdin in transgenic mice. Biliverdin is an intermediate in the enzymatic heme catabolism to bilirubin. As demonstrated with the *iRFP713* mouse, the NIR FP expression does not affect body weight, organ weight, blood indices, or reproductive performance. Under healthy conditions,

biliverdin is rapidly metabolized to bilirubin by biliverdin reductase A. Biliverdin reductase A knockout mice exhibit increased endogenous oxidative stress owing to arrest of the biliverdin conversion (Chen et al., 2018). The availability of biliverdin is higher in tissues with intensive heme catabolism, especially in the reticuloendothelial system, such as the spleen and liver. For example, in *iRFP713* mouse there is up to a three-fold variation in reporter fluorescence in different tissues (Tran et al., 2014), which may impose limitations for quantitative imaging or optogenetic manipulation with biliverdin-dependent probes.

Wide range of molecular biosensors

Intercellular communication and intracellular signaling are now accessible for long-term non-invasive monitoring with available transgenic mice expressing fluorescent biosensors. In a readout of the intrinsic ion fluxes or activation of signaling cascades, *in vivo* recordings with genomically integrated biosensors provides a stable signal, often with a higher amplitude and lower background, with the possibility for repeated measurements. In intact tissues, several cell types can contribute to the overall signaling cascade and metabolic pathway, and thus will fail to reproduce the same behavior *in vitro*. At the same time, certain requirements are posed on biosensors for application as a genomically integrated probe. These include high brightness, proper intracellular localization, sufficient dynamic range, and the on and off rate of the response.

Available transgenic mice now allow researchers to record second messengers, neurotransmitter turnover, membrane voltage, pH, chloride dynamic, and apoptotic events in living mice (Table S3). Mice encoding fluorescent biosensors for cAMP allowed recordings of real-time cAMP dynamics in response to GPCRs-mediated neuromodulatory inputs in the intact circuitry of the brain. These cAMP-encoded reporter (*CAMPER*) mice allow conditional expression in defined neuronal populations and were used to study the integration of GPCR signaling in neuronal circuits with subcellular resolution (Muntean et al., 2018). It was also demonstrated that baseline levels of cAMP differ across brain regions.

Several genetically encoded cyclic guanosine monophosphate (cGMP) biosensors have been reported (Honda et al., 2001; Nikolaev et al., 2006). A ratiometric cGMP indicator with EC50 of 500 nM/L was engineered using the Förster resonance energy transfer (FRET) pair of ECFP and EYFP linked in between with cGMP-binding domain of bovine cGMP-dependent protein kinase type I (Russwurm et al., 2007). cGMP binding decreases the ECFP/EYFP emission ratio (480 nm/535 nm) proportionally to the intracellular cGMP level. Characterization of this biosensor in cultured cells was soon followed by the generation of a *cGi500* transgenic mouse (Thunemann et al., 2013) (Table S3), which allows for studying cGMP responses in various tissues and organs.

Genetically encoded Ca^{2+} fluorescent biosensors that were initially engineered as a fusion of GFP and the M13 peptide sequence of myosin light-chain kinase, joined by Ca^{2+}-sensing calmodulin (GCaMPs), now include those bearing RFPs instead of GFP, which are preferable when combinations of reporters or optogenetic actuators are used. Now GCaMPs are presented by a variety of sensors with fast or slow responses, and different brightness for precise applications. A set of GCaMP6 biosensors (Chen et al., 2013) gave rise to *Thy1-GCaMP6s* and *Thy1-GCaMP6f* transgenic mice with stable neuronal expression of

the biosensors in the cortex and hippocampus. These mouse models enabled synchronous cortex-wide Ca^{2+} imaging of neural activity (Allen et al., 2017) and allowed to evaluate the capacity of individual neurons in the retrosplenial cortex to encode information for spatial navigation and context discrimination (Sun et al., 2021).

Mouse strains with genetically encoded calcium indicators, including FRET-based sensors such as yellow cameleon indicators, allow inducible and cell-specific expression for *in vivo* imaging. A fast variant of the GCaMP6 calcium indicator (GCaMP6f) (Chen et al., 2013) allows the detection of single action potentials with fast response kinetics and fast recording of neuronal activity in awake mice. Targeting of GCAMP6f to GABAergic neurons by crossing with $VGAT^{Cre}$ driver mice and subsequent two-photon imaging demonstrated that whisker stimulation suppresses the inhibitory interneurons (VGAT positive) in the primary auditory cortex of awaked mice, suggesting the important role of somatosensory inputs in auditory thalamocortical processing (Lohse et al., 2021). Studies on mice with GCAMP6f targeted to mitral cells of olfactory bulbs and olfactory somatosensory neurons demonstrated that the mammalian olfactory system has access to temporal features of odor stimuli in rapid odor fluctuation and allows mice to extract information about space from temporal odor dynamics (Ackels et al., 2021).

Genetically encoded voltage indicators (GEVIs), which comprise more structurally heterogeneous proteins, can be generated as FP with voltage-sensing domains, FRET pair of several FPs, and opsin-based or opsin-FP FRET pair sensors. A range of mice expressing GEVIs is now actively used in neuroscience for the optogenetic recording of action potential *in vivo* and in primary neurons. Mice expressing ArcLight, ASAP2, ASAP3, QuasAr2, and QuasAr3 are actively used to monitor action potentials and subthreshold depolarization in neurons (Jin et al., 2012) and enabled recordings of voltage dynamics in the mouse hippocampus *in vivo* (Adam et al., 2019).

Transgenic mice expressing chloride biosensor Clomeleon are used for imaging synaptic inhibition in various populations of neurons and in some more unique cases for studying GABA-mediated excitation in bipolar cells of the mouse retina (Duebel et al., 2006).

A transgenic mouse expressing glutamate biosensor iGluSnFr (Table S3) now opens the perspective for direct monitoring of its dynamics *in vivo*, which is also expected to happen for other neurotransmitters like GABA, glycine, serotonin, and dopamine, among others.

APPLICATIONS TO BASIC RESEARCH

Cell fate mapping for connectome analysis and developmental studies

Targeting imaging reporters to specific cell subpopulations in the tissue provides wide possibilities for tracking cell migration, differentiation, and functional input in tissue (Figure 4A). For these purposes, a simple reporter model uses the FP expressed under the control of cell-specific promoters to monitor cell migration to specific sites and/or differentiation. Thus, GFP expressed under the control of human CD68 antigen promoter/enhancer sequences in *hCD68-GFP* transgenic mice (Table S2) enables monitoring of monocyte trafficking to sites of inflammation and differentiation into macrophages *in situ*.

Activity-dependent promoters based on immediate-early genes, like FOS, now advance functional labeling of neuronal ensembles and functional brain mapping.

STochastic gene Activation with Regulated Sparseness knockin mice express mCherry (Shaner et al., 2004) and floxed mEYFP with the first 16 residues of Lyn kinase, which enables its myristoylation/palmitoylation and targeting to the plasma membrane. As mEYFP labels axonal and dendritic processes, it is suitable for tracing neuronal projections. The expression of mEYFP in specific subsets of neurons for brain mapping can be achieved with various Cre drivers.

Tracing the individual neurons based on their monosynaptically connected presynaptic partners allows for deciphering the contribution of certain neuronal inputs into external information sensing and processing in the central nervous system. Retrograde axonal transports implicated in the natural life cycle of neurotropic viruses, such as rabies, pseudorabies, and herpes simplex virus type-1, were used as transneuronal tracers in neuroscience (Wickersham et al., 2007). More recently, genetically modified pseudorabies virus type 1, vesicular stomatitis virus, lentivirus (Kato et al., 2011), and recombinant AAV-retro (Tervo et al., 2016) were introduced for efficient retrograde access to projection neurons.

The *Brainbow* technique (Cai et al., 2013) and *Confetti* mice encoding various FPs and Cre/lox system or Flp recombinase enable the labeling of individual cells with up to 100 different hues, sufficiently advancing studies on tissue development and regeneration (Choquet et al., 2020; Miao et al., 2019; Mizuhashi et al., 2018; Rios et al., 2014; Kurita et al., 2018), neurogenesis (Calzolari et al., 2015), tumorigenesis (Schepers et al., 2012), and connectome analysis. Brainbow multicolor fate mapping has now gone beyond an application in the brain and allows tracking events in embryology, developmental biology, and immunology (Tas et al., 2016). The application of this approach using $Aicda^{CreERT2/+}$ $Rosa26^{Confetti/Confetti}$ mice demonstrated that positive selection of B cells can take place in steady-state gut-associated germinal centers, which support targeted antibody responses to gut infections (Nowosad et al., 2020). During rapid turnover of germinal center B cells, this clone selection is tunable by the presence and composition of the microbiota. The targeting of *Brainbow* to retinal ganglion cells upon crossing with $Opn5^{Cre}$ mice demonstrated that opsin 5-dependent retinal light responses regulate vascular development in the postnatal eye (Nguyen et al., 2019).

Multispectral and combinatorial mosaic gene function analysis is now performed using inducible, fluorescent, and functional genetic mosaic analysis (*ifgMosaic* or *Dual ifgMosaic*) mice (Pontes-Quero et al., 2017). To map the expansion and arteriovenous fate of single endothelial cells the loss- and gain-of-function genetic mosaics were used with normal or altered Notch signaling. Chromatin-tagged FP in $iChr\text{-}Notch^{Tie2\text{-}Mosaic}$ embryos induces a mosaic of cells throughout the embryonic endothelium, with normal (H2B-Cherry$^+$), lower (dominant-negative MAML1$^+$ and H2B-EGFP$^+$), or higher (N1ICDP$^+$ and HA-H2B-Cerulean$^+$) Notch signaling. This approach demonstrates that the development of arteries depends on the timely suppression of endothelial cell-cycle progression and metabolism, a process that precedes arterial mobilization and complete differentiation (Luo et al., 2021).

Multiplexing for cell fate mapping in the brain can be applied using the quadruple-colored (*PrismPlus*) (Gaire et al., 2018) mouse line with EGFP fluorescence in microglia, YFP fluorescence in neurons, Cerulean fluorescence in oligodendrocytes and DsRed-Max (Strack et al., 2008) fluorescence in astrocytes. This animal model provides possibilities for *in vivo* two-photon microscopy in longitudinal studies, or for wide-view imaging modalities such as light-sheet microscopy, for characterization of the individual cellular population in different brain regions and their mobility toward relevant stimuli. The confirmed compatibility of this model with the advanced tissue clearing technique (CLARITY) (Chung et al., 2013) expands its application by three-dimensional imaging of intact large-scale tissue samples.

Multicolor *Confetti* reporter mice were adapted with the inclusion of an additional oncogenic cDNA following the tDimer2 RFP sequence, which allows an oncogene to be co-expressed specifically in RFP^+ clones (*Red2Onco* mouse). This mouse model is of value for understanding multiple aspects of the tumor microenvironment, oncogene-driven paracrine remodeling in tissue, with a local displacement of neighboring wild-type cells (Yum et al., 2021).

Defining patterns of protein expression

As the specificity of antibodies does not always allow to determine endogenous patterns of protein expression, the use of immunolabeling knockin mice with Cre or Flp recombinase expression under the control of endogenous promoters is a powerful instrument to define endogenous patterns of protein expression (Figure 4B). Moreover, with appropriate imaging techniques, such expression patterns can be registered *in vivo*. For example, the lack of specificity of available commercial antibodies against vitamin D receptor (VDR) prompted researchers to generate a new knockin mouse strain with Cre recombinase expression under the control of the endogenous VDR promoter (VDR^{Cre}). Cre activity in the VDR^{Cre} mouse brain was highly overlapping with endogenous VDR mRNAs, allowing for visualization of VDR-expressing cells and characterizing their functions (Liu et al., 2021). VDR profiling in the brain can shed light on its role in brain development and neuroprotective responses (Kasatkina et al., 2020).

Another approach allows tracking the patterns of endogenous protein knockout upon crossing the Cre driver to the mouse bearing floxed allele. A reporter mouse model with floxed alleles of both the mouse GAD1 and GAD2 (glutamate decarboxylase 1 and 2) genes, in addition, has floxed tdTomato reporter. This model allows the detection of specific cellular populations with Cre-mediated knockdown of GAD1 and GAD2 and revealed the critical roles of GABA in the control of feeding and metabolism (Meng et al., 2016).

Monitoring organelle and protein trafficking

Among the prospective applications of mice expressing imaging reporters, we discuss the ability to track not only individual cells in multicellular organisms but also to study the dynamics of organelles and proteins within the cell (Figure 4C). The progress in this field relies on both the implementation of new fluorescent reporters, including photoactivatable variants, and the simultaneous development of techniques for high spatial resolution imaging.

The main strategy for tracking cellular organelles is the design of fluorescent reporter fusions with organelle-specific markers. Several reporter mice were generated for tracking mitochondria dynamics and fusion. These mice express FP fused with cytochrome *c* oxidase subunit VIIIa, which is a terminal enzyme of the mitochondrial respiratory chain. The fusion of the cytochrome *c* oxidase subunit VIIIa with enhanced cyan FP (Heim et al., 1994) was used to selectively image mitochondrial dynamics in the mouse nervous system *in vivo* and acute explants (Misgeld et al., 2007). Dendra2 green/red photoswitchable monomeric FP derived from coral *Dendronephthya sp* (Gurskaya et al., 2006) enables local labeling of organelles to track their dynamics. Mice bearing the floxed mitochondrial-specific version of Dendra2, $PhAM^{floxed}$ (photo-activatable mitochondria) allow measurements of mitochondrial fusion and transport. When the subpopulation of mitochondria is exposed to 405 nm light, green fluorescence is irreversibly switched to red.

Cells use the lysosome system to degrade and recycle aged and damaged organelles, misfolded proteins, and internalized infection agents. The diversity of cellular phagosomes at different stages of maturation can be visualized using mice encoding FP fusion with the microtubule-associated protein 1 light chain 3α. Transgenic mice expressing fuse of microtubule-associated protein 1 light chain 3 alpha with the red FP under the control of MYH6 promoter allow measuring cardiac autophagic flux *in vivo* (Martinez et al., 2011).

Extracellular vesicles, such as microvesicles, exosomes, and apoptotic bodies, are now recognized as important mediators of intercellular communication. They differ in size, biogenesis, and cargo. The labeling of circulating extracellular vesicles and subsequent detection by immunohistochemistry, FACS analysis, or *in vivo* was shown on transgenic *CD9-EGFP* mice (Neckles et al., 2019).

Dynamics of second messengers and ion fluxes

Genomically encoded chloride, Ca^{2+}, cAMP, cGMP, and voltage sensors expressed in transgenic mice allow for studying signaling cascades in excitable and non-excitable tissues.

Transgenic *cGi500* mouse (Table S3) encoding cGMP indicator (Russwurm et al., 2007) enables the visualization of cardiovascular cGMP signals in real time and is a useful tool to study vascular cGMP dynamics associated with vasodilation *in vivo* (Thunemann et al., 2013). Megakaryocyte/platelet-specific expression of cGi500 was achieved by crossing the cGi500-L2 strain (with floxed cGi500 transgene) to the platelet-specific $PF4^{Cre}$ driver strain and allowed demonstrating that shear stress significantly potentiates nitric oxide-induced cGMP generation in pre-activated platelets and results in platelet inhibition at later stages of thrombus formation (Wen et al., 2018).

Transgenic mice expressing GEVIs together with light-gated ion channels, like channelrhodopsins, are used for all-optical electrophysiology (Figure 4D). Multiple parameters can be registered simultaneously with the proper combination of the reporters for multiplexed applications. For simultaneous light-controllable stimulation and voltage recording, bicistronic vectors for the co-expression of blue-shifted channelrhodopsin actuator (CheRiff-EGFP) and NIR Archaerhodopsin-derived voltage indicator QuasAr1-mOrange2 (Optopatch1) or QuasAr2-mOrange2 (Optopatch2) were engineered (Hochbaum

et al., 2014). The later construct was used to generate an *Optopatch* mouse, which enables Cre-dependent expression (also called *Floxopatch*) (Lou et al., 2016), for the simultaneous optical stimulation and measurement of membrane voltage. The engineering of improved NIR Archaerhodopsin-derived voltage indicator QuasAr3 led to the generation of the *Optopatch3* (*Ai155*) mouse (Adam et al., 2019), which displays a higher signal-to-noise ratio in genetically targeted all-optical electrophysiology recordings. A fusion of the GCaMP6f indicator with QuasAr2 conditionally expressed in *CaViAr* mouse provides an opportunity for simultaneous recording of Ca^{2+} dynamics and membrane voltage changes in excitable cells.

TRANSLATIONAL STUDIES AND DRUG DISCOVERY

Assessment of host-pathogen interactions

In vivo and *ex vivo* fluorescence imaging are essential for the development and screening of new drug and vaccine candidates. Transgenic mice with encoded imaging reporters enable tracking of pathogen tropism, specific receptors enabling entry to the cells, immune invasion, involvement of the specific T and B cell subpopulations, and host response for efficient search of new drug targets and screening of their selective effectors.

The fusions of FP and receptors involved in the recognition molecules for multiple pathogens, including viruses, bacteria, protozoa, and fungi, are useful reporters to monitor the activation of innate immunity. A range of mice strains was generated to express fluorescent reporters together with recognition receptors, implicated in innate immunity and adaptive immunity to pathogens and resolution.

An important part of the innate immune response is the assembly of inflammasome triggered by cytosolic activated pattern recognition receptors, which recognize pathogen-associated or host-derived danger-associated molecular patterns. A reporter mouse encoding the fluorescent adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (*ASC-citrine* mouse) is used for characterization of inflammasome assembly. The formation of assembled inflammasome complexes (specks) was shown in different tissues and at distinct phases of viral and bacterial infection. It has been demonstrated that the speck-forming cells exhibited pyroptosis and extensive release of specks to the extracellular milieu *in vivo* (Tzeng et al., 2016).

Reporters can be targeted to dendritic cells with CD11c promoter (Table S1) to study their antigen-presenting function during the initiation of an adaptive immune response. Mice encoding EYFP targeted to dendritic cells enabled studies of the steady-state and mature dendritic cells in various regions of the lymph nodes (Lindquist et al., 2004). Fluorescent dendritic cells were imaged with two-photon microscopy in the *CD11c-EYFP* mouse in lymph nodes at depths of up to 300 μm.

Markers in cell replacement and transplantation

FPs and their fuses may serve as a transplantation marker for embryonic and adult tissue. Imaging reporters expressed in transgenic mice make animals a valuable xenograft/transplant host. Transgenic mouse models, including *Confetti*, *Brainbow*, *PrismPlus*, and

iRFP713, are evolving to the platform for cell subtype classification and multiplexed quantification of protein markers in transplants for tracking graft development and integration. This expands the screening of new compounds targeting the cell division, differentiation, adhesion, and migration as well as immune response during graft rejection.

Fluorescent reporter mice expressing mRFP1 (Campbell et al., 2002) in early development (Zhu et al., 2005) can be used for cell population visualization during embryogenesis or transplantation, as well as for studies on chimeric mice and multiplexed imaging along with green and cyan FPs. A high level and ubiquitous expression of the reporter in *mRFP1* mouse does not interfere with cell morphology, developmental potential, viability, or fertility.

Pharmacological targeting of receptor-specific functions

Genomically encoded designer receptors exclusively activated by designer drugs (DREADDs), based on engineered G-protein-coupled receptors, provide the possibility to analyze the contribution of certain signaling pathways to the physiological response. The advantages of DREADDS include low baseline activity, insensitivity to endogenous (natural) ligands and minimal, if any, off-target activity of pharmacologically inert ligands. In line with these opportunities, a fusion of imaging reporter to DREADDs enables tracking of cell-specific expression of the tool and locates positive cells for manipulation.

DREADD derived from human M3 muscarinic receptor (hM3D) was modified for binding synthetic agonist clozapine-N-oxide. Downstream signaling with the involvement of the Gαq subunit triggers the PLC-mediated generation of PIP_2 and IP_3 and induces Ca^{2+} release from intracellular stores and enhances neuronal excitation.

The *in vivo* visualization and activation of genetically defined cells were accessed using mice, which enable Flp/Cre recombinase-dependent targeting of DREADDs. Genomically encoded hM3Dq/mCherry fusion protein enabled somatodendritic localization and reproducible expression in mice (Sciolino et al., 2016). Studies confirmed that activation of hM3Dq is sufficient to dose-dependently and noninvasively control the activity of diverse cellular populations to analyze their specific functions at any developmental stage (Sciolino et al., 2016).

Inhibitory DREADD that induces the canonical Gi/o pathway was generated from human M4 muscarinic receptor (hM4Di). Signaling via this receptor inhibits adenylate cyclase, decreases cAMP production and provokes K^+ efflux, which results in cell-autonomous hyperpolarization and decreased neuronal firing. Mouse model allowing conditional hM4Di and mCherry expression following the sequential steps of recombination was used as a noninvasive tool for mapping neuron function. Targeting this inhibitory DREADD to serotoninergic neurons of the lower brainstem demonstrated the involvement of these cells in life-sustaining respiratory and thermoregulatory networks. Studies supported the role of these cells as central respiratory chemoreceptors capable of regulating the downstream respiratory network and lung ventilation in an attempt to restore a normal arterial pH/PCO_2 (Ray et al., 2011).

CONCLUSIONS

Stable transgene expression, targeting expression to various tissues, undisrupted tissue microenvironment, and the possibility for long-term imaging make transgenic mice the advanced research tools. Recent *in vivo* studies demonstrate that transgenic mice expressing imaging reporters provide unique information in the areas where tissue connectivity and overall homeostatic regulation are essential for studied processes. As proteins for fluorescent imaging and genetically encoded biosensors continue to evolve, we expect the generation of new transgenic animals and mice, in particular, to expand our knowledge on poorly studied signaling cascades and metabolic pathways.

New reporter mouse lines can be generated based on recently developed genetically encoded biosensors for neurotransmitters (Leopold et al., 2019) like gamma-aminobutyric acid (Marvin et al., 2019), dopamine (Sun et al., 2018), norepinephrine (Feng et al., 2019), glycine (Zhang et al., 2018b), and serotonin (Wan et al., 2021). Mice expressing genetically encoded cell-eath indicators (Linsley et al., 2021) can advance studies of early cell death triggers in neurodegeneration. The genetically encoded sensor system, which enables the multicolor and signal-amplified imaging of endogenous RNAs, was already introduced (Zhou et al., 2021) and its application in mice can soon be expected.

We anticipate that biosensors for other neuromediators including aspartate, adenosine, histamine, and endocannabinoids, which are highly required for *in vivo* studies, will be engineered and introduced in transgenic animals. *In vivo* dynamics for most of the second messengers and hormones is still inaccessible owing to the absence of specific genetically encoded probes. Therefore, protein engineering in this direction may serve as a trigger for new possibilities in the non-invasive monitoring of various signaling pathways.

Recently engineered protein kinase A activity reporter (ExRai-AKAR2) (Zhang et al., 2021) could be introduced in transgenic mice for *in vivo* monitoring of protein kinase A signaling. Several other kinase reporters with a large dynamic range are suitable for *in vivo* imaging and could be used to develop new mouse models. A kinase translocation reporter approach based on a nucleocytoplasmic shuttling of phosphorylated reporters enables the measurement of single-cell activation of multiple signaling pathways simultaneously with high temporal resolution (Regot et al., 2014). A separation of phases-based activity reporter of kinase also provides an easy approach to quantitatively image dynamic kinase signaling in living animals (Zhang et al., 2018a).

Intravital imaging still has a limited spatiotemporal resolution, which could be improved in the future by refinement of imaging optics. Recently, an advanced two-photon fiberscopy with a deep learning algorithm has been applied to image the arousal-induced activity changes in layer 2/3 pyramidal neurons in the primary motor cortex of freely behaving mice, providing opportunities to define the neural basis of behavior (Guan et al., 2022). Combining such techniques with NIR imaging probes could expand their potential and provide access to deeper brain regions.

In optical detection, the light penetration to deep brain structures and developing organs remains problematic, frequently requiring *in utero* imaging with intravital windows (Huang

et al., 2020). Ultrasound examination or MRI, in contrast, cannot use the advantages of transgenic organisms with optical reporters. This limitation can be overcome by using photoacoustic imaging. In this regard, a transgenic mouse with the knocked-in bacterial phytochrome BphP1 from *Rhodopseudomonas palustris*, which binds biliverdin as a chromophore and reversibly photoswitches between two absorbing states (680 nm and 740 nm), provides a substantial advance (Kasatkina et al., 2022). This mouse allows both a NIR light-induced gene transcription activation with AAV-delivered BphP1 binding-partner QPAS1 and a differential photoacoustic tomography using BphP1 photoswitching. This *loxP-BphP1* mouse enables Cre-dependent temporal and spatial targeting of BphP1 expression and non-invasive deep tissue imaging.

The future development of sophisticated techniques for non-invasive imaging, in particular photoacoustic imaging (Yang et al., 2019; Li et al., 2021; Liu et al., 2018), will go hand-in-hand with the generation of new imaging probes and reporter mice. Such mutual progress should facilitate the use of conditional transgenic mouse models expressing novel NIR probes with tumor markers, intracellular antigen receptors and nanobodies, and boost the development of deep tissue imaging applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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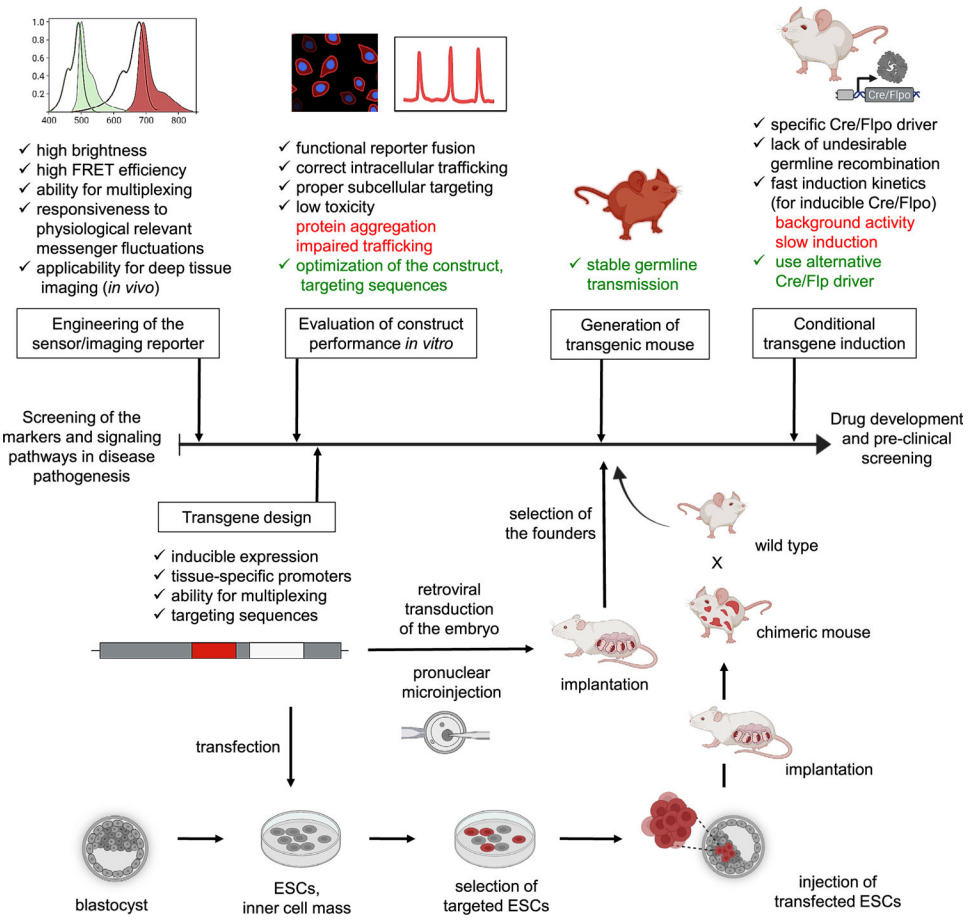

Figure 1. Workflow depicting major steps starting from the engineering of an optical reporter to the generating of a transgenic mouse

Possible challenges and their solutions are shown. The design of the transgene comprises the selection of optical reporter and the targeting strategies. Conditional transgene induction is determined by the transgene design and the selection of Cre/Flp driver.

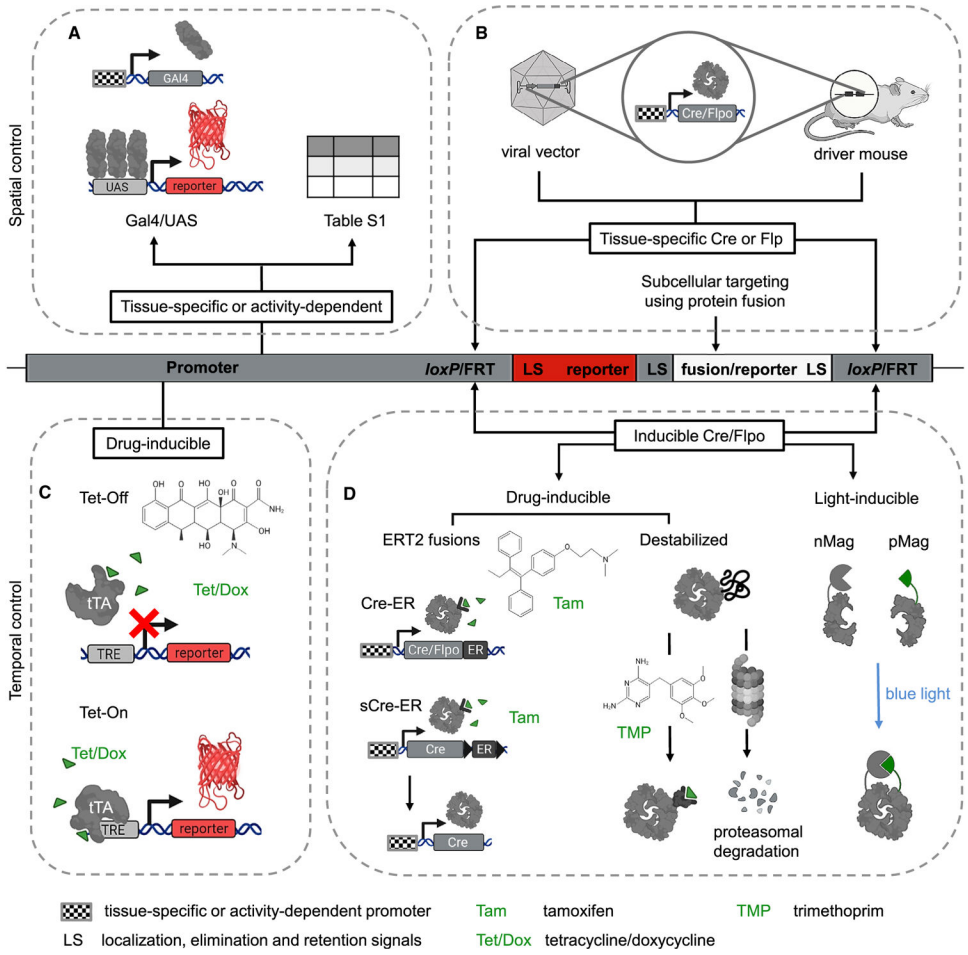

Figure 2. Approaches for spatial and temporal control of reporter expression in transgenic mice

(A) Targeting the transgene expression using tissue-specific/activity-dependent promoters or tissue-specific Gal4/UAS (Upstream Activation Sequence) system.

(B) Using Cre/*loxP* system or Flippase for conditional reporter expression in progeny or following viral-mediated delivery. FP fusions and localization signals enable further subcellular targeting.

(C) Tet response element (TRE) upstream of a minimal promoter is recognized by a Tet-controlled transactivator (tTA) (Tet-On/Off system) and is used for inducible reporter expression.

(D) Photoactivatable and destabilized Cre/Flp. Fusion of Cre with a tamoxifen-sensitive form of the estrogen receptor (ER) and self-cleaved Cre (sCre-ER) provide the control for both spatial and temporal reporter expression, and the possibility to switch the Cre to a constitutively active form. Destabilized Cre/Flp and light-inducible Cre enable chemogenetic and optogenetic control of floxed reporters.

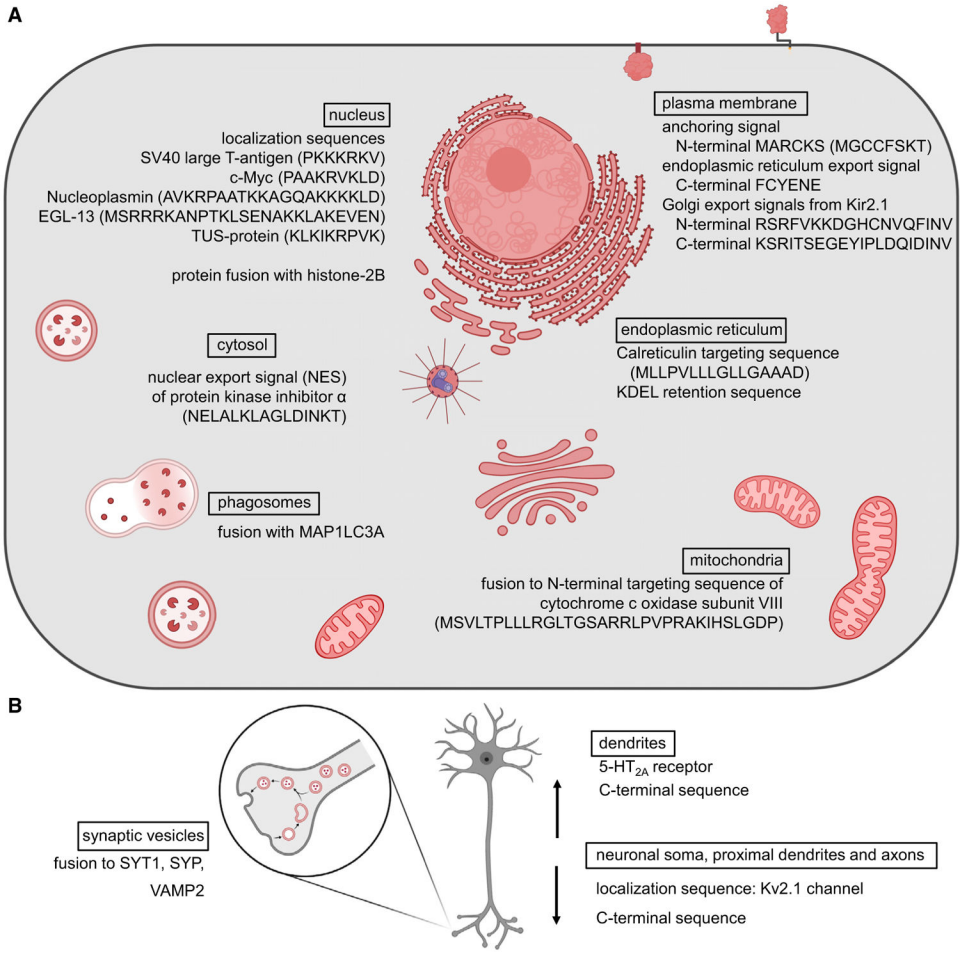

Figure 3. Sequences for subcellular targeting of transgene expression in mice

(A) Localization sequences and nuclear export sequences located on N- and C-termini, or reporter protein fusion provide targeting of the synthesized protein to the specific cellular compartments or anchor the reporter on the plasma membrane.

(B) FP fuses can be designed for targeting reporters to the proximal and distal cellular compartments and/or specialized organelles (i.e., synaptic vesicles). MARCKS, myristoylated alanine-rich C-kinase substrate. MAP1LC3A, Microtubule-associated proteins 1A/1B light chain 3A.

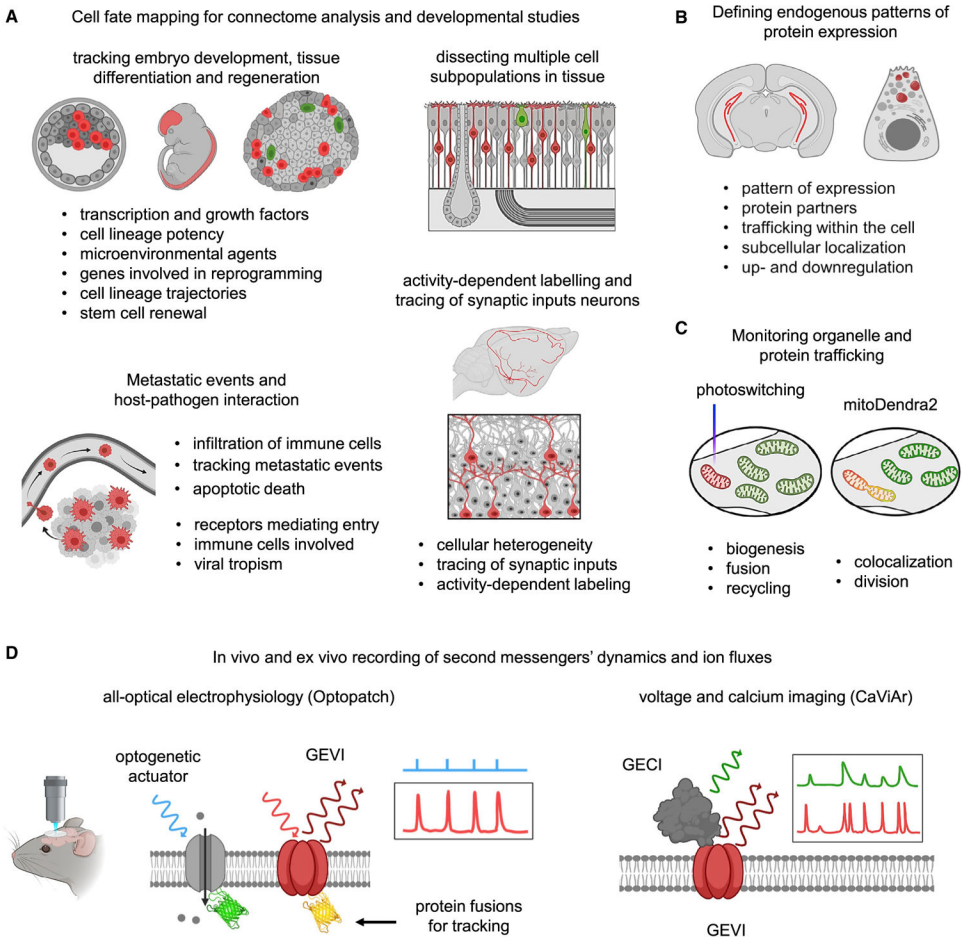

Figure 4. Applications of transgenic mice with tissue-specific expression of optical probes

(A) Cell-specific reporter expression from the early embryonic stage throughout the lifespan provides the tools for cell fate mapping during embryogenesis, tissue differentiation and regeneration. Distinct cellular subpopulations can be dissected within the tissue following experience-dependent activation, or based on monosynaptic circuit tracing.

(B and C) Using specific promoters and reporter protein fusions for tracking endogenous patterns of protein expression (B) or organelle dynamic both *in vitro* and *in vivo* (C).

(D) Transgenic mice expressing combinations of imaging reporters enable longitude studies of ion fluxes, second messenger generation and simultaneous recording of several functional parameters (Ca^{2+}, membrane voltage) during intrinsic or ontogenetically induced neuronal activity.