Topic Introduction

Generation and Screening of Transgenic Mice with Neuronal Labeling Controlled by *Thy1* Regulatory Elements

Petar Marinković, Leanne Godinho, and Thomas Misgeld

Major progress has been made using in vivo imaging in mice to study mammalian nervous system development, plasticity, and disease. This progress has depended in part on the wide availability of two-photon microscopy, which is capable of penetrating deep into scattering tissue. Equally important, however, is the generation of suitable transgenic mouse models, which provide a “Golgi staining”-like labeling of neurons that is sparse and bright enough for in vivo imaging. Particularly prominent among such transgenic mice are the so-called *Thy1*-XFP mice (in which XFP stands for any fluorescent protein) that are used in numerous studies, especially to visualize spine plasticity in the cortex and remodeling in peripheral synapses. New generations of *Thy1*-XFP mice are now being generated at a high rate, and these have allowed previously difficult experiments to become feasible. Moreover, with easy access to core facilities or commercial providers of pronuclear injections, generating simple *Thy1* transgenic mice is now a possibility even for small laboratories. In this introduction, we discuss the *Thy1* regulatory elements used to generate transgenic lines with neuronal labeling. We provide a brief overview of currently available *Thy1* transgenic mice, including lines labeling neuronal organelles or reporting neuronal function.

INTRODUCTION

Thanks to Golgi’s *reazione nera*, the value of high-contrast labeling of stochastic subsets of nerve cells has been apparent since the dawn of modern neuroscience (Ramon y Cajal 1904). However, translating these early achievements into a method to observe nerve cells in vivo over time has required another century of technological development. With the advent of two-photon microscopy and major advances in fluorescent protein development, such studies have now become feasible. Although many different approaches to such studies have been explored (Young and Feng 2004), most recent chronic in vivo imaging studies of nerve cells in the central nervous system (CNS) and peripheral nervous system (PNS) have taken advantage of transgenic mice that express high levels of fluorescent proteins in subsets of nerve cells driven by murine *Thy1* regulatory elements (Lichtman and Sanes 2003; Misgeld and Kerschensteiner 2006; Pan and Gan 2008; Holtmaat and Svoboda 2009; Wilt et al. 2009). A collection of such transgenic mice (*Thy1*-XFP mice) was first described by Feng et al. (2000), and since then, numerous studies have taken advantage of these original transgenic lines. In addition, many new variants of such mice have been engineered (see *Thy1* Lines below). The central role that *Thy1*-XFP mice have come to play in some areas of in vivo imaging and the plethora of lines now available motivate our attempt in this introduction to provide background information about the *Thy1* promoter itself, the available *Thy1* lines, and the generation of new ones. Moreover, as core facilities that generate transgenic founder mice for a reasonable fee are now available at most major

Adapted from *Imaging in Neuroscience: A Laboratory Manual* (ed. Helmchen and Konnerth). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top087668

research institutions, generating new *Thy1* transgenic animals is an option even for relatively small laboratories.

The *Thy1* Expression Vector

The *Thy1* expression vector (Fig. 1) is derived from a gene that encodes a small immunoglobulin superfamily member, *Thy1* (GeneID 21838; Ingraham et al. 1986), which is anchored via glycosylphosphatidylinositol (GPI) to the plasma membrane. Endogenously, murine *Thy1* is widely expressed on numerous cell types, including immune cells and neurons (Morris 1992). As one of the first discovered surface markers of specific immune cell subsets, effective antibodies became available early. In addition to immunological studies, this allowed detailed exploration of the expression of *Thy1* in the nervous system (Morris 1985). Studies of transcriptional regulation followed suit, revealing the strong and broad expression in nerve cells controlled by *cis*-acting elements within the locus. This allowed tailoring expression toward neuronal specificity, while largely suppressing immune cell expression (Vidal et al. 1990). Together, these features made the *Thy1* gene the source of well-characterized pan-neuronal regulatory elements and an obvious choice for the creation of neuron-specific transgenic mice (Caroni 1997). Originally, *Thy1*-derived regulatory elements were used to express disease- and remodeling-associated genes (Aigner et al. 1995; Andra et al. 1996). Because the *Thy1* gene is up-regulated only postnatally in most neuronal populations, the *Thy1*-driven gene expression in these studies allowed circumventing undesirable developmental effects, without resorting to complex conditional gene-expression strategies. In these studies, the strong variation in

FIGURE 1. The murine *Thy1* locus and the *Thy1* cloning vector. (A) Schematic map of the endogenous *Thy1* locus. (B) *Thy1* cloning vector. The critical restriction sites for cloning are indicated. Important features within the plasmid are annotated. (Modified from Ingraham et al. 1986; Vidal et al. 1990, and Caroni 1997.)

expression levels among different classes of neurons, but also, more importantly, among individual neurons of the same population, was already noted (Caroni 1997). Although this variation represents a potential problem for studies that use *Thy1* regulatory elements to establish pan-neuronal expression of transgenes to test for their biological effects, it has proven an unexpected blessing for studies that aim at revealing neuronal morphology using in vivo imaging.

Stochastic Subset Labeling

In 2000, Feng and colleagues took advantage of the strong but variable neuronal expression driven by *Thy1* regulatory elements to label neurons in vivo (Feng et al. 2000). They described what have become known as *Thy1*-XFP transgenic mice, a set of 25 transgenic lines with individual expression patterns of green fluorescent protein (GFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), or DsRed. Among these originally described lines were some in which the majority of neurons endogenously expressed the *Thy1* gene, as well as others that showed a striking Golgi-like labeling pattern (Fig. 2). These *Thy1*-XFP lines of the first generation are still widely in use, and they can be easily obtained from The Jackson Laboratory (<http://www.jax.org>). They have been featured in numerous seminal in vivo imaging studies of developmental, physiological, and pathological neuronal plasticity (e.g., Grutzendler et al. 2002; Trachtenberg et al. 2002; Gan et al. 2003; Walsh and Lichtman 2003; Xu et al. 2009; Yang et al. 2009).

One of the most useful features of *Thy1*-driven expression of fluorescent proteins has proven to be its baffling sensitivity to chromosomal position effects. This results in a wide variety of expression patterns in different founders that originate from pronuclear injection of the same *Thy1* construct (Fig. 3). Expression varies with respect to both the neuronal subpopulations that are labeled and the percentage of cells within a population that expresses the transgene. Although few general rules have held up without exception, certain neuron populations still tend to show expression in most *Thy1* transgenic lines. These neuronal populations typically give rise to long-distance projections (e.g., motor neurons, large-diameter dorsal root ganglion neurons, retinal ganglion cells, or cerebellar mossy fibers), but some cells with local projections (e.g., dentate gyrus granule cells in the hippocampus) are also typically labeled. In many lines, subpopulations of excitatory cortical neurons (e.g., pyramidal cells in the cortex or hippocampal region CA1) are labeled to a variable degree. Less commonly, local interneurons, Purkinje cells, climbing fibers, or small-diameter sensory neurons are labeled. However, many unexpected labeling patterns can be found, even in nonneuronal cells. For example, lines have been reported with expression in astrocytes or terminal Schwann cells (Livet et al. 2007). As a general rule, transgene expression becomes detectable during the first postnatal week in most *Thy1* lines in accordance with the developmental profile of endogenous *Thy1* expression (Morris 1985). However, *Thy1*-XFP lines show a wide range of variation with regard to expression onset as well. For example, *Thy1*-YFP¹⁶ (Feng et al. 2000) shows strong expression in motor neurons as early as embryonic day 13 (Lichtman and Sanes 2003).

A rather surprising feature of *Thy1*-driven expression is the fact that within a seemingly homogeneous population of neurons (e.g., the motor neurons of a given muscle's motor pool) individual neurons can show strong labeling, while in adjacent cells, such labeling is undetectable. This does not represent a slight difference in the labeling intensity, such that the detection sensitivity creates an arbitrary cutoff above which some brighter neurons would be scored as labeled, whereas dimmer labeling would be scored as absent. Indeed, in some of the so-called subset *Thy1* lines, labeling of the few expressing cells is brighter than the brightest cells in any of the "full" lines. These labeling patterns are characteristic for a given *Thy1*-XFP line and are heritable, and numerous distinct lines that differ in labeling density, intensity, and expressed fluorophore are now available. With regard to subset labeling and the related nomenclature, it should be noted that the original *Thy1*-XFP lines were categorized as full or subset mostly based on the percentage of motor neurons labeled in a limited number of motor pools because the completeness of motor neuron labeling is easily scored by counterstaining synaptic sites in the periphery (Fig. 2). This nomenclature does not imply that a full line necessarily shows complete labeling of any other neuronal population, or even of all motor neurons in all motor pools.

FIGURE 2. Two *Thy1* lines with typical sites of expression. (A,D,G) Line *Thy1-YFP¹⁶*, a typical full line. (B,E,F,H) Line *Thy1-YFP^H*, a subset line. (C) In situ hybridization of endogenous expression pattern (modified from Allen Mouse Brain Atlas; contrast inverted). Confocal analysis of parasagittal sections (A,B) shows that even a full line only labels subsets of most neuronal populations, albeit in this case, at a significantly higher frequency than in the subset line. (D,E,F) Higher power views confirm this for the cerebellum. In-line *Thy1-YFP¹⁶* mossy fibers and parallel fibers are densely labeled, whereas line *Thy1-YFP^H* shows sparse subset labeling in these axon populations (see isolated mossy fiber rosette in F, left). In addition, however, line *Thy1-YFP^H* very occasionally also harbors labeled Purkinje cells (F, middle; inset shows spiny dendrites at full resolution) and even nonneuronal Bergmann glia (F, right). In the neuromuscular system, full lines, such as *Thy1-YFP¹⁶*, show complete overlap between preterminal axon labeling (yellow) and bungarotoxin-labeled postsynaptic sites (magenta label; G). In subset lines, such as line *Thy1-YFP^H*, motor units can be traced instead (H; inset shows a labeled nonneuronal cell). Lines were originally described in Feng et al. (2000). Scale bars: in A, 1 mm (for A,B); in D, 100 μ m (for D,E); in F (left), 5 μ m; in F (center, right), 50 μ m (inset, 2.5 mm); in G, 50 μ m (for G,H). Images acquired on an FV1000 confocal system (Olympus) with 4X/0.13-NA air (A,B), 20X/0.85-NA oil (D,E), and 60X/1.42-NA oil (remaining panels) objectives. (C, Modified, with permission, from the Allen Mouse Brain Atlas 2010 [<http://mouse.brain-map.org/viewImage.do?imageId=68400987>]; Lein et al. 2007.)

Still, a general tendency exists that lines with subset labeling in one population of neurons also show subset labeling of other neuronal populations. However, all *Thy1* lines lack labeling of some neurons or neuronal subsets; hence, *Thy1*-driven expression should never uncritically be accepted as pan-neuronal. One unfortunate feature of *Thy1*-driven subset expression is its developmental dynamics. In many subset lines, the density of labeling increases as the animal ages, which can be an impediment in studies aimed at longitudinal reidentification of the same neurons. Moreover, at least based on anecdotal observations, the density of subset labeling in individual animals of a given line seems to be influenced by its genetic background (and even gender). As a consequence, different substrains of the original *Thy1*-XFP lines now likely exist, and in planning studies based on these animals, such variation should be taken into consideration.

The exact reasons for the subset expression found in *Thy1*-XFP lines remain unknown. Generally, this variability is ascribed to positional effects relating to insertion site and copy number of the transgene. Moreover, effects akin to position effect variegation could explain the subset character

FIGURE 3. Spectrum of expression patterns found in a typical screen of founders. This figure shows the full spectrum of expression patterns found in the spinal cord of the MitoMice (*Thy1*-mitoCFP, cyan; Misgeld et al. 2007). Labeling density ranges from almost complete (e.g., lines B,C) to extremely sparse (line H). Subset labeling is not restricted to *Thy1*-transgenic lines. Lines Y and Z were generated using neuron-specific enolase-derived regulatory elements to express mitoYFP (yellow). (Nse lines courtesy of R.W. Burgess, The Jackson Laboratory; images were taken in collaboration with M. Kerschensteiner, Ludwig-Maximilians University, Munich.) First image shows Nissl-like fluorescent stain (magenta) of a matched spinal cord section (cst, corticospinal tract; dc, dorsal column; vh, ventral horn). Scale bar, 250 μ m. Images acquired on an FV1000 confocal system (Olympus) with a 4 $\times$ 0.13 air objective.

of some lines (Feng et al. 2000). No study to date, however, has systematically explored this question or taken advantage of subset *Thy1* lines to define new neuronal cell classes based on the integration loci of the *Thy1* transgenes.

THY1 LINES

Overview of Available *Thy1* Lines

Since the description of the first *Thy1*-XFP lines by Feng et al. (2000), numerous additional sets of *Thy1*-driven transgenic lines that express a wide variety of conventional and functionalized fluorescent proteins have been reported. Initially, efforts aimed at widening the spectrum of available labeling patterns and colors (second generation). Additionally, attempts were initiated to systematically control subset labeling using the cre-loxP recombination system (third generation). In parallel, with the advent of well-characterized mammalian organelle targeting sequences and the proliferation of fluorescent-protein-based functional reporter constructs, numerous *Thy1* lines were generated that permit in vivo assaying of organelle distribution and transport, intraneuronal ion concentrations, neuronal spiking activity, and synaptic release. An overview of these lines, and the corresponding references, is provided in Table 1. Many of these lines are now available either through The Jackson Laboratory (<http://www.jax.org>) or directly from the laboratories that generated them. Considerable challenges for laboratories that generate *Thy1* lines are the financial and infrastructural requirements for line maintenance. Moreover, expression patterns in many of the reported lines have not been systematically documented, at least not in centralized public domain databases. Therefore, centralized deposition of frozen embryos or sperm, in addition to public domain documentation of expression patterns, should become a future priority.

Generating *Thy1* Lines

The generation of new *Thy1* transgenic lines generally consists of five steps: (1) engineering and characterization of the desired fluorescent reporter protein, (2) cloning of the reporter protein into the *Thy1* vector, (3) linearization and purification of the new *Thy1* construct, (4) pronuclear injection to generate founders, and (5) screening of founder progeny to establish transgenic lines. Procedures for Steps 2 and 3 above are described in **Generation of *Thy1* Constructs for Pronuclear Injection** (Marinkovi et al. 2015a) and procedures for Step 5 are described in **Generation of Tissue Sections for Screening *Thy1* Mouse Lines** (Marinkovi et al. 2015b) and **Imaging Acute Neuromuscular Explants**

TABLE 1. *Thy1* lines described in the literature

Reference	Name	Purpose	Fluorophores	Available at The Jackson Laboratory?
First-generation <i>Thy1</i> -XFP lines				
Feng et al. 2000	<i>Thy1</i> -XFP	Assessment of neuronal morphology	CFP, GFP, RFP, YFP	Y
Second-generation <i>Thy1</i> -XFP lines				
De Paola et al. 2003	<i>Thy1</i> -membXFP	Neuronal plasma membrane labeling	GFP	
Hirrlinger et al. 2005	<i>Thy1</i> -RCFP	Assessment of neuronal morphology	Coral FPs	
Third-generation <i>Thy1</i> -XFP lines				
Buffelli et al. 2003	<i>Thy1</i> -STOP-YFP	Subset labeling and neuronal Cre reporter	YFP	Y
Livet et al. 2007	Brainbow	Multicolor labeling of neurons	CFP, GFP, RFP, YFP	Y
Organelle <i>Thy1</i> -XFP lines				
De Paola et al. 2003	<i>Thy1</i> -synaptophysin-XFP	Synapse localization and SV trafficking	GFP, RFP	
Umemori et al. 2004	<i>Thy1</i> -synaptophysin-YFP	Synapse localization and SV trafficking	YFP	Y
Südhof 2008 ^a	<i>Thy1</i> -syntaxin1a-YFP	Synapse localization and SV trafficking	YFP	Y
Han et al. 2005	<i>Thy1</i> -synaptotagmin-YFP	Synapse localization and SV trafficking	CFP	Y
Südhof 2008 ^b	<i>Thy1</i> -VAMP2-CFP	Synapse localization and SV trafficking	CFP	Y
Misgeld et al. 2007	MitoMouse (<i>Thy1</i> -mitoCFP)	In vivo assessment of mitochondrial transport	CFP, YFP	Y
Functionalized <i>Thy1</i> -XFP lines				
Berglund et al. 2006	<i>Thy1</i> -clomeleon	Ratiometric measurement of chloride levels	CFP/YFP	
Heim et al. 2007	<i>Thy1</i> -CerTN-L15	FRET measurement of calcium levels	Cerulean/citrine	
Arenkiel et al. 2007	<i>Thy1</i> -Chr2-YFP	Optogenetic control of neuronal firing	YFP	Y
Young et al. 2008	SLICK	Cre-mediated recombination in labeled neurons	YFP	Y
Li et al. 2005	<i>Thy1</i> -synapto-pHluorin	Synaptic transmitter release	YFP	
Tabares et al. 2007	<i>Thy1</i> -synapto-pHluorin	Synaptic transmitter release	YFP	
Wyatt et al. 2008	<i>Thy1</i> -synapto-pHluorin	Synaptic transmitter release	YFP	

CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein; RFP, red fluorescent protein variants (e.g., DsRed1, Tdimer2); FPs, fluorescent proteins; FRET, fluorescence resonance energy transfer.

^aMGD Direct Data Submission [MGI Ref ID J:132968].

^bMGD Direct Data Submission [MGI Ref ID J:131425].

from *Thy1* Mouse Lines (Marinković et al. 2015c). Step 1 (generation and characterization of desired reporter proteins) depends critically on the exact reporter being studied. Therefore, a general protocol cannot easily be provided. Often, fluorescent reporters are first characterized in simple cell lines, such as human embryonic kidney (HEK) cells. However, in our experience, a difficult problem remains that the performance in transgenic mice is often not easily predictable on this basis. For example, many fluorescent proteins that appear unproblematic in cell culture show aggregation when expressed at high levels in vivo. This represents a particular problem with fusion proteins, in which in vivo functionality can often not be proven. One approach is to cross the transgenic animal into a mutant strain that carries a deletion of the gene under study. Phenotypic rescue would suggest intact function of the fusion protein, provided expression is properly targeted. Such a stringent assay, however, is rarely possible and requires considerable breeding efforts. Methods that allow transient overexpression in mice (such as in utero electroporation) can provide valuable hints with regard to in vivo performance of a new reporter. Given these challenges, plus the cost and effort involved in making transgenic animals, it often is advisable to err on the side of caution and use transgenic fluorescent proteins that have been tested in vivo already, rather than use the product of the latest improvement in fluorescent protein engineering.

After the desired fluorescent protein reporter sequence is cloned into the *Thy1* vector (see **Generation of *Thy1* Constructs for Pronuclear Injection** [Marinković et al. 2015a]), it must be sent to a facility specializing in pronuclear injection (Step 4). After successful pronuclear injection, the transgenic facility will generally transfer a set of polymerase chain reaction (PCR)-confirmed founders (i.e., animals that, on tail biopsy, were found to contain the *Thy1* vector sequences). To screen for functional expression, we cross founders with commercially obtained inbred mice (typically C57BL/6).

Animals of the F₁ generation are screened at 3 wk of age, when *Thy1*-driven expression has typically reached high levels. In most cases, more than half of the PCR-confirmed founders have protein-expressing offspring. So as not to jeopardize lines during screening, it is important to always keep sufficient numbers of PCR-confirmed breeders until the screening process for a given line is complete.

For screening, we typically prepare acute nerve–muscle explants (see **Imaging Acute Neuromuscular Explants from *Thy1* Mouse Lines** [Marinković et al. 2015c]) in parallel to transcardial perfusion (see **Generation of Tissue Sections for Screening *Thy1* Mouse Lines** [Marinković et al. 2015b]) because fixation can weaken fluorescence and create misleading autofluorescence. In addition, this allows the first functional tests of the expressed biosensors. Positive lines are then established by further crossing into the in-bred strain, and the lines are maintained by PCR-based selection of positive animals. Once the desired level of backcrossing to a defined genetic background is reached, animals can be homozygized for the transgene by brother–sister inbreeding.

Because screening, for cost and space reasons, is typically performed in the F₁ generation, changes in expression patterns are possible in future generations because of multiple insertions of the transgene that segregate. Similarly, changes in genetic background can affect expression patterns. Therefore, new lines should be regularly checked for stability of the desired expression patterns, and care should be taken to indicate generation numbers when first describing new lines.

Fluorescent proteins can modulate protein function or even exert toxic effects when fused to functional domains (Han et al. 2005) or when expressed at very high levels (Bridge et al. 2009). Therefore, careful functional studies of fusion proteins before and after transgenic mouse generation are necessary. Generally, it is advisable to maintain lines that show lower levels of fluorescent reporter protein in addition to highly expressing lines.

ACKNOWLEDGMENTS

We thank Martin Kerschensteiner, Ludwig-Maximilians University Munich, Rob Burgess, Jackson Laboratory, and Jeff Lichtman, Harvard University, for collaboration on obtaining the pictures shown in Figure 3. We also acknowledge the kind permission of the Allen Brain Institute to use Figure 2C. Work in our laboratory is supported by the Alexander-von-Humboldt foundation, the TUM Institute for Advanced Study, and the Bundesministerium für Bildung und Forschung (BMBF).

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Cold Spring Harbor Protocols

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Cold Spring Harb Protoc; doi: 10.1101/pdb.top087668

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