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Labeling neurons *in vivo* for morphological and functional studies

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Increasingly sophisticated strategies for labeling cells *in vivo* are providing unprecedented opportunities to study neurons in living animals. Transgenic expression of genetically encoded reporters enables us to monitor changes in neuronal activity in response to sensory stimuli, and the labeling of single neurons with fluorescent proteins allows the dynamics of neuronal connectivity to be observed in transgenic animals over periods ranging from minutes to months. Advances in transient labeling techniques such as viral infection and electroporation provide a rapid means by which to analyze neuronal gene function *in vivo*. These new approaches to labeling, manipulating and imaging neurons in intact organisms are transforming the way in which the nervous system is studied.

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Abbreviations

GFP	green fluorescent protein
MARCM	mosaic analysis with repressible cell marker
RNAi	RNA interference
XFP	generic term for fluorescent protein

Introduction

The nervous system is composed of a tightly packed, heterogeneous mixture of billions of cells, and this complexity poses a major challenge to neurobiologists. The ability to label small subsets of neurons facilitates the study of neuronal morphology and function. Historically, the application of methods such as Golgi staining to fixed tissues has helped to lay the foundations of modern neuroscience. Today, increasingly sophisticated techniques enable isolated neurons to be labeled and imaged *in vivo*, and these new approaches are being used to give us a glimpse of how the brain works in living animals.

The simplest of these methods rely on the use of diffusible marker molecules, such as dyes or fluorescent proteins, to label axons and dendrites, and thus to reveal neuronal morphology. More complicated markers can be used to tag specific subcellular structures, such as synapses, or to report physiological parameters of neurons, such as membrane depolarization, synaptic activity or ion concentration. Although much can be learned by simply observing wild type neurons *in vivo* [1], other approaches, in which cells are labeled while gene function is simultaneously manipulated, facilitate elucidation of the molecular mechanisms that underlie particular neuronal properties.

In this review, we discuss both transgenic and transient methods for labeling neurons *in vivo*, focusing particularly on strategies for labeling small subsets of cells and highlighting some remarkable applications of such techniques that have been reported in the past two years.

Labeling neurons in transgenic animals

The development of fluorescent proteins as genetically encoded vital dyes has markedly increased the popularity of labeling neurons through transgenesis. Advantages of transgenic labeling over transient transfection approaches include the fact that it is non-invasive and it can provide stable labeling of neurons in a pattern that is reproducible from animal to animal.

Flies and worms

Transgenic labeling and imaging of neurons *in vivo* was pioneered in the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*. Ten years ago, green fluorescent protein (GFP) was first used to label sensory neurons in transgenic *C. elegans* [2]. In flies, enhancer trap screens have also enabled specific classes of neurons to be labeled by genetically encoded markers [3] and further refinements of this approach have led to the development of methodologies such as the 'Flp-out' system, which enables random labeling of isolated cells within a defined population of neurons [4,5]. In this system the site specific recombinase Flp is under the control of a heat shock promoter, and single cell labeling is achieved by subjecting flies to a mild heat shock which induces a low level of Flp expression. Flp mediated recombination then switches on expression of a GFP-based marker in a small number of neurons. More recently, neuronal labeling in flies has reached a new level of sophistication with the development of the mosaic analysis with repressible cell marker (MARCM) system, which uses mitotic recombination to make small clonal populations of cells that are homozygous for a mutation and simultaneously labeled with GFP [6]. This system permits mutant neurons to be analyzed in the context of an otherwise wild

type animal [7,8] and is discussed in detail in an accompanying review in this issue.

In vivo applications of genetically encoded probes for physiological activity have also achieved most success in flies and worms. For example, three groups have recently expressed GFP-based Ca^{2+} sensors in neurons of the fly olfactory system to analyze odor-evoked maps of neuronal activity [9,10••,11]. Similarly, neural activity in the fly olfactory system has also been imaged using synapto-pHluorin [12]. Synapto-pHluorin is a pH-sensitive GFP variant that is fused to the synaptic vesicle protein VAMP2 (vesicle-associated membrane protein 2) and functions as a probe for synaptic vesicle release [12,13]. In addition, Ca^{2+} imaging of presynaptic terminals at neuromuscular junctions in fly larvae has been used to study active zone density during synaptic strengthening [14].

Mice

Compared with neuronal labeling in invertebrates, transgenic labeling of neurons in mice presents many challenges. Many promoters fail to give robust expression of transgenes, whereas others label large populations of cells, thereby precluding the imaging of individual neurons. Labeling of single isolated olfactory neurons was achieved by using olfactory receptor promoters to drive reporter gene expression [15]. This approach was successful because in the olfactory system a given olfactory receptor is only expressed in a relatively small subset of olfactory neurons that are scattered throughout the nasal epithelia, which enables isolated cells to be labeled and imaged. However, this system proved to be the exception rather than the rule.

A different strategy for achieving sparse labeling of neurons takes advantage of a phenomenon called 'position effect variegation', which causes line-to-line variations in transgene expression [16]. By generating many lines of mice expressing GFP (or one of the spectral variants of GFP that we collectively term XFP) under the control of a modified Thy1 promoter, it is possible to identify transgenic lines in which small subsets of neurons are labeled brightly [17]. Of particular interest are the few lines that express XFP in isolated single neurons in accessible regions of the nervous system such as the neocortex, olfactory bulb and the neuromuscular junction (Figure 1). The combination of Golgi-like labeling with a genetically encoded vital stain makes these mice a valuable tool with which to visualize the dynamic processes of neural development and remodeling in a living organism.

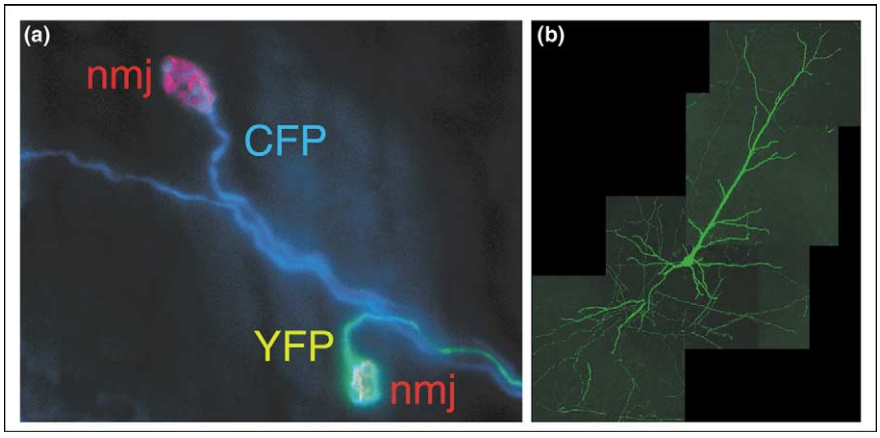
Several recent *in vivo* imaging studies using Thy1–XFP transgenic mice illustrate the advantages of transgenic labeling. Lichtman and co-workers [18,19,20••] crossed transgenic lines expressing spectrally distinct fluorescent proteins in individual motor neurons in order to observe the elimination of multiple inputs to neuromuscular junctions, an event that occurs postnatally. In the most recent of these studies the use of multicolor labeling facilitated an examination of competition for junctions between pairs of innervating neurons [20••]. Thy1–XFP mice have also been used to study dendritic structure in the brain over periods ranging from minutes to months. For example, two-photon microscopy has been used to observe the dynamics of dendritic spines in XFP-labeled pyramidal cells in the cerebral cortex [21,22], as well as to examine dendritic structure in mitral and tufted neurons in the olfactory bulb [23].

An alternative strategy for achieving sparse labeling of neurons in transgenic mice is to express a reporter gene, such as *GFP*, under the control of an inducible form of Cre recombinase. With this system, administration of an activating ligand results in the dose-dependent labeling of cells. By using low doses of ligand, the labeling of isolated neurons can be achieved [24,25] in a manner which is analogous to the mild heat shock induced labeling in the 'Flp-out' system. This approach should allow lineage analyses to be carried out if the recombinase is activated in neural progenitor cells. In the future, variations of this strategy should permit simultaneous labeling and gene knockout in clonally related neurons in mice in a manner similar to the MARCM system in the fly.

Another exciting area of development is the generation of mice that express fluorescent probes of physiological function [26]. For example, mice expressing Clomeleon, a fluorescent-protein-based Cl^- sensor [27], have been generated and have facilitated the study of synaptic inhibition in a population of neurons [28]. Another example is the expression of synapto-pHluorin in olfactory neurons in mice [29••]. *In vivo* imaging of the olfactory bulb in these mice showed that neuronal activity, as measured by synapto-pHluorin fluorescence, increases in individual glomeruli in response to odorant stimulation.

The goal of many transgenic experiments is to label a particular neuronal population of interest. A limitation of traditional transgenic approaches is that the relatively small promoter regions that are usually used do not always give rise to the expression pattern expected for the endogenous gene. These problems can often be overcome by using bacterial artificial chromosomes to generate transgenic mice [30]. This strategy enables very large regions of regulatory sequence to be included in the transgenic construct and has been recently used to express GFP from the parvalbumin promoter to label parvalbumin-positive interneurons *in vivo* [31]. This has permitted whole-cell recordings to be made from identified interneurons *in vivo* by a novel technique called 'two-photon targeted patching' [32]. In the future, transgenic labeling based on bacterial artificial chromosomes will be a very useful technique for dissecting the functions of specific populations of neurons.

Figure 1



Labeling subsets of neurons with fluorescent proteins in transgenic mice. Transgenic mice are generated by using the regulatory element of the mouse Thy1 gene to drive the expression of green fluorescent protein (GFP) or one of its spectral variants, yellow, cyan or red fluorescent protein (collectively termed XFP), in projection neurons [17]. Owing to position effect variegation, many lines of transgenic mice express XFP in only small subsets of neurons. **(a)** Live image of the neuromuscular junction (nmj) taken from a double transgenic mouse that expresses cyan fluorescent protein (CFP) in all motor neurons and yellow fluorescent protein (YFP) in a small subset of motor neurons. Acetylcholine receptors are stained with rhodamine-conjugated α-bungarotoxin to mark the synaptic sites. Labeling different subsets of motor neurons with spectrally different fluorescent proteins facilitates the study of neuronal competition during synapse elimination at the neuromuscular junction [19,20••]. Image courtesy of J Lichtman. **(b)** Image showing that isolated single neurons in the cortex are brightly labeled in their entirety. The Golgi-like labeling of live neurons in accessible regions of the brain, such as the neocortex and olfactory bulb, enables repeated identification and imaging of the same neuron, the same dendrites and the same spines over a period of days to months [21–23]. Image courtesy of S Danzer.

Transient approaches to labeling neurons

There are several methods for acutely introducing labeling molecules (either dyes or genetically encoded markers) into neurons *in vivo*. The main advantages of such transient approaches are the decrease in experimental turnover time, the ability to control the location and timing of labeling, and the ability to carry out other manipulations simultaneously in the labeled cells. Potential drawbacks include the invasive nature and toxicity of some methods, variability in labeling between experiments, and the inaccessibility of many regions of the nervous system to transient labeling, especially in adult animals.

Physical methods

Physical methods for labeling neurons are especially powerful in accessible preparations such as the optic tectum of *Xenopus* tadpoles or zebrafish larvae. Two recent studies illustrate the utility of this approach for studying different aspects of neural development *in vivo*. Neill *et al.* [33•] injected DNA encoding a fluorescent postsynaptic marker protein (PSD95–GFP) into zebrafish embryos at the 1–4-cell stage and carried out time-lapse imaging of the dendritic arbors of labeled tectal neurons. This approach enabled them to define the relationship between synapse formation and dendritic branch dynamics and provided evidence that synapse formation can direct dendritic arborization. The second study used ionophoretic loading of fluorescent dyes to observe the branching of retinal ganglion cell axons and determined how the stability of axonal branches is influenced by correlated patterns of neuronal activity in *Xenopus* tectum [34].

Electroporation

Electroporation involves the application of an electric pulse that temporarily disrupts the plasma membrane and forces DNA or other molecules into cells as a result of their charge. A key advantage of electroporation is the ease with which two or more expression constructs can be simultaneously introduced into cells. This approach facilitates the introduction of dominant-negative or constitutively active constructs into cells that are labeled through the coexpression of a marker protein, usually GFP [35].

In vivo electroporation has been used widely in the neural tube of developing chick or mouse embryos [36,37]. More recently, it has found broader applications in the retina, cerebellum and cerebral cortex of early postnatal and adult rodents [38–41]. The spatial control of electroporation has been taken to the extreme with the development of single-cell electroporation techniques that facilitate the labeling of individual neurons [42–44]. For example, using single-cell electroporation, Sin *et al.* [45] defined a role of Rho GTPases in the regulation of dendritic plasticity driven by visual stimulation in GFP-labeled optic tectal neurons.

The coelectroporation of two constructs has also been used to combine RNA interference (RNAi) with GFP labeling to 'knockdown' the expression of target genes in

identified neuronal populations [39,40,46,47•]. Using this approach, Konishi *et al.* [39] identified a novel role of the anaphase-promoting complex in axonal outgrowth and parallel fiber patterning in the cerebellum, and Matsuda and Cepko [40] knocked down the expression of two transcription factors required for photoreceptor development, thereby replicating the phenotype seen in knockout mice lacking these transcription factors. A similar strategy has been used in embryonic chick spinal cord to test candidate genes identified in a subtractive hybridization screen for factors involved in commisural axon guidance [47•]. The resulting rapid *in vivo* readout of gene function facilitated identification of the most promising genes and suggests that this strategy of combining RNAi with neuronal labeling is suitable for applications in functional genomics.

Viral vectors

Many viral vectors are now available that can be used for gene transfer into neurons [48]. Among the most promising for *in vivo* applications are the lentiviruses, because lentiviral vectors have low toxicity and can provide long-term expression of reporter genes. Lentiviruses containing neuron-specific promoters have been used to label neurons specifically *in vivo* [49], and lentiviral systems for RNAi have also been developed [50•,51•]. In addition, lentiviruses can infect a wide variety of species, and lentiviral infection of embryos results in germline transmission of the integrated virus, thereby providing a means with which to express reporter genes stably in species other than mice [50•,51•,52]. Taken together, these developments suggest that lentiviruses could be used both to label neurons and to silence genes *in vivo* in a relatively routine manner.

Conclusions

Several landmark applications of *in vivo* neuronal labeling have been reported over the past few years. The continuing development of novel labeling strategies combined with advances in imaging techniques suggests, however, that we are just seeing the tip of the iceberg. In the near future, it should be possible to make genetic manipulations within sparsely labeled neurons in mice, similar to the MARCM analysis methodologies that are used in flies. It is also expected that improved GFP-based reporters of neuronal physiology will gradually find more *in vivo* applications. Furthermore, electroporation-based assays will be used to whittle down long lists of genes arising from genome-wide expression profiling experiments to only those genes that are functionally relevant. One can even envisage the use of viral vectors or electroporation to carry out RNAi-based genetic screens for neuronal function in species in which traditional forward genetics has not been previously feasible.

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