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# Simultaneous neuron- and astrocyte-specific fluorescent marking

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ABSTRACT

Systematic and simultaneous analysis of multiple cell types in the brain is becoming important, but such tools have not yet been adequately developed. Here, we aimed to generate a method for the specific fluorescent labeling of neurons and astrocytes, two major cell types in the brain, and we have developed lentiviral vectors to express the red fluorescent protein tdTomato in neurons and the enhanced green fluorescent protein (EGFP) in astrocytes. Importantly, both fluorescent proteins are fused to histone 2B protein (H2B) to confer nuclear localization to distinguish between single cells. We also constructed several expression constructs, including a tandem alignment of the neuron- and astrocyte-expression cassettes for simultaneous labeling. Introducing these vectors and constructs *in vitro* and *in vivo* resulted in cell type-specific and nuclear-localized fluorescence signals enabling easy detection and distinguishability of neurons and astrocytes. This tool is expected to be utilized for the simultaneous analysis of changes in neurons and astrocytes in healthy and diseased brains.



## 1. Introduction

Recent neuroscience research has largely focused on the analysis of changes in specific subtypes of brain cells. This focus has led to many insights into the structural alterations in central nervous system disorders, including major depression, Huntington's disease, an autism [1–4]. These findings strongly suggest that many psychiatric diseases feature a major disruption in the normal composition of brain cell populations. It is therefore important to comprehensively investigate which cell types are affected in disease states; however, techniques for simultaneously labeling and imaging of multiple cell types have not yet been adequately developed. In the central nervous system, neurons and astrocytes are likely to be the most affected cell types, making them interesting targets for more detailed analysis.

Cell type-specific marker mice are already widely used and available for a variety of cell subsets, for example EGFP-marker mice are available from the GENSAT project [5]; however, the fluorescent markers expressed by these reporter lines all have the same color and only allow analysis of a single cell type at a time. To investigate distinct cells or cell types at the same time, multicolor labeling systems have also been developed. The Brainbow

*Abbreviations:* EGFP, enhanced green fluorescent protein; GHE, GfaABC$_1$D-H2B-EGFP; GFAP, glial fibrillary acidic protein; H2B, histone 2B protein; hSyn, human synapsin 1; hSyn-HT, hSyn-H2B-tdTomato; MAP2, microtubule-associated protein 2; NRSE, neuron-restrictive silencer element; PBS, phosphate-buffered saline; Scg10, superior cervical ganglion 10; SNHT, Scg10-NRSE-H2B-tdTomato; SNHT-ins-GHE-ins, Scg10-NRSE-H2B-tdTomato-ins-GfaABC$_1$D-H2B-EGFP-ins.

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mouse is based on the Cre recombinase-controlled expression of a random combination of fluorescent proteins, resulting in Cre-expressing cells that are labeled with various different colors [6]. This system can distinguish individual neighboring cells by their color; thus it is excellent for neuronal tracing approaches but is not applicable for encoding certain cell types by defined colors. A Prism mouse line aims to fulfill this purpose by causing the specific expression of yellow, cerulean, and red fluorescent proteins in oligodendrocytes, astrocytes, and neurons, respectively [7]; however, this transgenic mouse shows an abnormal hyperactive behavioral phenotype due to the overexpression of a nonspecific gene in one of the bacterial artificial chromosomes used for the transgenic mouse generation, rendering this mouse difficult to use as a wild-type reference for psychiatric disease models. Moreover, the fluorescent proteins in the Prism mice and those in most other marker mouse lines are expressed in the cytoplasm; thus, the distinguishability of neurons from their surrounding neighbors in brain areas with highly dense cell populations is difficult. One possible solution to this problem is the targeting of fluorescent signals to the cell nucleus, which makes easy position assignment possible.

In the present study, we attempted to develop a cell type-specific method of marking neurons and astrocytes with nuclear restricted fluorescence signals.

## 2. Materials and methods

### 2.1. Vector construction

The constructed cell type-specific expression vectors are shown in Fig. 1. The-116 bp fragment of a neuron-restrictive silencer element (NRSE) fragment and the corresponding complementary sequence were synthesized (Epoch Life Science Inc., TX, USA; sequence in Table S1), annealed, and used as an insert for ligation into the *Bam*HI site of the ptdTomato-N1 vector (Clontech Laboratories Inc., CA, USA) with the In-Fusion HD cloning kit (Clontech Laboratories Inc.). The-2007 bp sequence of a superior cervical ganglion 10 (Scg10) promoter was amplified from rat genomic DNA with the sense primer 5′-GAGGACAATGGA AGTTGTGTTG-3′ and the antisense primer 5′-TTTAGCCATTGTAGG GATGTG-3′, then digested with *Bgl*II and *Sac*II and inserted into the NRSE-containing ptdTomato-N1 vector at the corresponding restriction sites to yield scg10-NRSE-tdTomato. The H2B sequence was excised from pCAG-H2B-tdiRFP-IP (a gift from Dr. Michiyuki Matsuda, Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University) using *Bgl*II and *Bam*HI and inserted into the *Bam*HI site between the NRSE sequence and the tdTomato start codon of scg10-NRSE-tdTomato to yield Scg10-NRSE-H2B-tdTomato (SNHT).

The 681-bp sequence of a compact glial fibrillary acidic protein (GFAP) promoter (GfaABC$_1$D) and the corresponding complementary sequence were synthesized (Epoch Life Science Inc.; Table S1), annealed, and inserted into the *Pac*I and *Xho*I sites of the pEGFP-N1 vector (Clontech Laboratories Inc.) to yield GfaABC$_1$D-EGFP. Then, the H2B sequence was inserted into GfaABC$_1$D-EGFP in the same manner as described above to generate GfaABC1D-H2B-EGFP (GHE). The 5 × tandem insulator sequence (ins [8]) and the corresponding complementary sequence were synthesized, annealed, and inserted into the *Kpn*I and *Sac*I sites of pBluescript II KS(+) (Stratagene, CA, USA) to yield ins-pBluescript, which was then re-digested using *Sal*I and *Spe*I for subsequent insertion upstream of GfaABC$_1$D in GfaABC$_1$D-H2B-EGFP to yield ins-GfaABC$_1$D-H2B-EGFP. A four-part In-Fusion ligation was performed with the combined ins-GfaABC$_1$D-H2B-EGFP cassette excised by *Spe*I and *Afl*II, two linker fragments synthesized as short double-stranded fragments (Epoch Life Science Inc.; Table S1), and the ins-pBluescript vector linearized using *Afl*II and *Not*I, resulting in the GfaABC$_1$D-H2B-EGFP cassette flanked by an insulator sequence on either side to yield the ins-GfaABC$_1$D-H2B-EGFP-ins-pBluescript. Finally, the Scg10-NRSE-H2B-tdTomato cassette excised using *Nhe*I and *Afl*II from SNHT was inserted into the corresponding sites upstream of the first insulator sequence in ins-GfaABC$_1$D-H2B-EGFP-ins-pBluescript to generate Scg10-NRSE-H2B-tdTomato-ins-GfaABC 1D-H2B-EGFP-ins (SNHT-ins-GHE-ins). The human Synapsin 1 (hSyn) promoter sequence was excised from the hSyn-GFP-pLS vector (a gift from Dr. Tohru Matsuki at the Institute for Developmental Research, Aichi Human Service Center) using *Xba*I and *Eco*RI and inserted into the multicloning site of pBluescript to yield hSyn-pBluescript. The H2B-tdTomato cassette from Scg10-NRSE-H2B-tdTomato was inserted into hSyn-pBluescript using the In-Fusion HD cloning kit to generate hSyn-H2B-tdTomato-pBluescript (hSynHT).

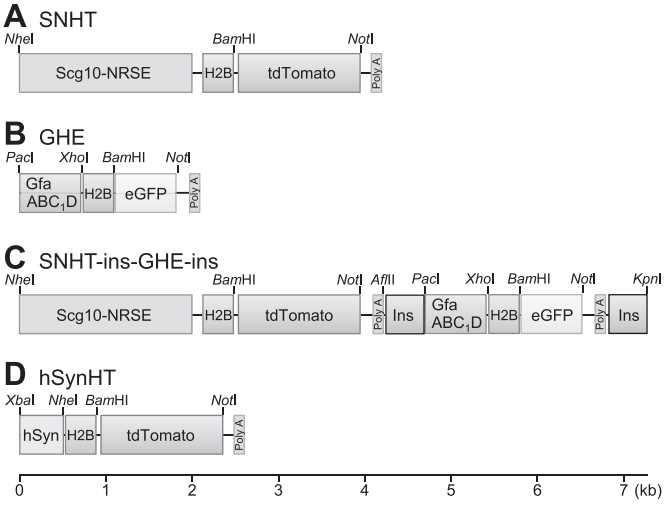


**Fig. 1. Schematic construction of cell type-specific expression vectors**. A single expression vectors with the neuron-specific promoter Scg10-NRSE (SNHT) (A), a single expression vectors with the astrocyte-specific promoter GfaABC1D (GHE) (B), a tandem expression cassette vector with Scg10-NRSE and GfaABC1D (SNHT-ins-GHE-ins) (C), and a single expression vector with the neuron-specific promoter hSyn (hSynHT) (D) were designed. Nuclear localization was achieved by fusing the fluorescent proteins to the histone 2B (H2B) protein. The restricted digestion sites used for cloning are indicated above the respective vectors. ins, insulator sequence; poly A, polyadenylation site from Simian Virus 40.

### 2.2. Cloning of lentivirus vectors

The human Synapsin 1 (hSyn) promoter sequence was excised from the hSyn-GFP-pLS vector using *Xba*I and NheI and inserted into the multicloning site of the RFP-QM512B vector from the SpaQ Cumate Switch system (System Biosciences, CA, USA) to generate hSyn-RFP-QM512B. The RFP sequence was then exchanged with the H2B-tdTomato cassette from Scg10-NRSE-H2B-tdTomato by subcloning using the In-Fusion HD cloning kit for site-specific integration to generate hSyn-H2B-tdTomato-QM512B.

The GfaABC$_1$D-H2B-EGFP cassette was first amplified by PCR with the sense primer 5′-GTCACTCGTTTAATTAAGAACATATCC TGGTGTGG-3′ and the antisense primer 5′- GTAATACGACTCACTA-TAGGGC-3′ to add a *Spe*I restriction site upstream of GfaABC$_1$D. The amplified product was then digested using *Spe*I and *Not*I and inserted in place of hSyn-H2B-tdTomato at the corresponding sites of the hSyn-H2B-tdTomato-QM512B vector to generate GfaABC1D-H2B-EGFP-QM512B.