Journal of Neuroscience Methods 240 (2015) 77–88

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Neurons can be labeled with unique hues by helper virus-free HSV-1 vectors expressing Brainbow

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HIGHLIGHTS

- Brainbow creates hundreds of hues by combinatorial expression of fluorescent proteins.
- In Brainbow mice, multiple neurons contain the same hue, as many neurons are labeled.
- HSV-Brainbow labels small numbers of neurons, and their axons, with unique hues.
- Specific axons can be matched to specific neuronal cell bodies, based on hue.

ARTICLE INFO

Article history:
Received 25 August 2014
Received in revised form
11 November 2014
Accepted 12 November 2014
Available online 20 November 2014

Keywords:
Brainbow
Fluorescent protein
Cre-mediated recombination
Hue
Axon projection
Herpes simplex virus vector

ABSTRACT

Background: A central problem in neuroscience is elucidating synaptic connections, the connectome. Because mammalian forebrains contain many neurons, labeling specific neurons with unique tags is desirable. A novel technology, Brainbow, creates hundreds of hues by combinatorial expression of multiple fluorescent proteins (FPs).
New method: We labeled small numbers of neurons, and their axons, with unique hues, by expressing Brainbow from a helper virus-free Herpes Simplex Virus (HSV-1) vector.
Results: The vector expresses a Brainbow cassette containing four FPs from a glutamatergic-specific promoter. Packaging HSV-Brainbow produced arrays of seven to eight Brainbow cassettes, and using Cre, each FP gene was in a position to be expressed, in different cassettes. Delivery into rat postrhinal (POR) cortex or hippocampus labeled small numbers of neurons with different, often unique, hues. An area innervated by POR cortex, perirhinal (PER) cortex, contained axons with different hues. Specific axons in PER cortex were matched to specific cell bodies in POR cortex, using hue.
Comparison with existing methods: HSV-Brainbow is the only technology for labeling small numbers of neurons with unique hues. In Brainbow mice, many neurons contain the same hue. Brainbow-adeno-associated virus vectors require transduction of the same neuron with multiple vector particles, confounding neuroanatomical studies. Replication-competent Brainbow-pseudorabies virus vectors label multiple neurons with the same hue.
Conclusions: Attractive properties of HSV-Brainbow include each vector particle contains multiple cassettes, representing numerous hues, recombination products are stabile, and experimental control of the number of labeled neurons. Labeling neurons with unique hues will benefit mapping forebrain circuits.

Abbreviations: AAV, adeno-associated virus; AP, anterior–posterior; β-gal, β-galactosidase; DV, dorsal–ventral; DMEM, Dulbecco's modified minimal essential medium; EmGFP, emerald green FP; EBFP2, enhanced blue FP-2; FBS, fetal bovine serum; FPs, fluorescent proteins; HSV-1, Herpes Simplex Virus; IR, immunoreactivity; IVP, infectious vector particles; ML, medial-lateral; PER, perirhinal; PAG, phosphate-activated glutaminase; POR, postrhinal; PRV, pseudorabies virus; VGLUT1, vesicular glutamate transporter-1.

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http://dx.doi.org/10.1016/j.jneumeth.2014.11.009
0165-0270/

1. Introduction

Nervous system functions, ranging from physiological homeostasis to advanced cognitive tasks, are encoded in specific neuronal circuits, composed of specific neurons and synapses. Thus, elucidating all the synaptic connections in a specific nervous system, the connectome, is a central problem in neuroscience, and a challenging task, due to the large numbers of neurons and synapses in a nervous system. Nonetheless, some remarkable progress has been made. The entire connectome for the nematode *Caenorhabditis elegans* was determined by electron microscopy (White et al., 1986). *C. elegans* contains a relatively small number, ~300, neurons; in contrast, mammalian nervous systems, and especially the mammalian forebrain, are considerably more complex. The mouse forebrain contains 10^3–10^4 types of neurons (Sugino et al., 2006), a single rat neocortical column contains ~7500 neurons (Peters and Jones, 1984), and human neocortex contains ~10^9 synapses per cubic mm (Alonso-Nanclares et al., 2008). Of note, specific projections in mammalian nervous systems have been mapped using a wide range of techniques (Luo et al., 2008; Zaborszky et al., 2006), including electron microscopy, classical anterograde and retrograde tracers, genetic tracers (Lo and Anderson, 2011), genetic synaptic markers (Feinberg et al., 2008), and viruses (Ekstrand et al., 2008). Specific circuits have been mapped, such as the connections between the different primate neocortical areas that process visual information (Felleman and Van Essen, 1991). However, efficient, high-resolution mapping of forebrain circuits, at the level of individual neurons and axons, remains a challenge.

One challenge for track tracing technologies is to uniquely label individual neurons. Many track tracing technologies label multiple neurons with the same tag, and obtain resolution from serial section reconstruction and process tracing. These technologies range from Golgi staining, as pioneered by Cajal, to specific genetic track tracers, such as expression of a single fluorescent protein (FP). Specific modifications to these techniques support labeling neurons with several different tags, such as several fluorescent dyes or two FPs; these approaches are advantageous for many studies, but, nonetheless, label multiple neurons with the same tag. Recently, a powerful new technology, Brainbow, was developed that creates hundreds of hues by combinatorial expression of different FPs (Livet et al., 2007); a Brainbow cassette contains two to four different FPs, Cre-mediated recombination probabilistically determines which FP is expressed, and an array of Brainbow cassettes results in expression of one specific combination of FPs, from hundreds of potential combinations, or hues. Nonetheless, in Brainbow transgenic mice (Livet et al., 2007), multiple neurons are labeled with the same hue, because the number of labeled neurons far exceeds the number of hues produced by Brainbow; this issue exists even with advantageous Brainbow array integration site, Cre-driver mouse line, and Brainbow promoter. Thus, in Brainbow mice, serial section reconstruction and individual axon tracing would be required to map the projections of individual neurons. To address these issues, mice with Brainbow recombination limited to a specific neuron type might be advantageous; Brainbow mice with serotonergic-specific Cre-expression have been reported (Weber et al., 2009), but analogous approaches in the forebrain will likely label large numbers of neurons, resulting in a challenging axon mapping problem. Further, a number of second-generation Brainbow constructs in transgenic mice have been explored to increase expression levels, and better control Cre-mediated recombination; nonetheless, the use of Brainbow mice for mapping forebrain circuits remains problematic (Cai et al., 2013).

Neurons might be labeled with unique hues by expressing Brainbow in a limited number of neurons, using a virus vector. However, as a single Brainbow cassette is several kb, an array of five to ten Brainbow cassettes will challenge, or exceed, the capacity of a number of virus vectors. Moreover, a procedure is required to obtain Cre-mediated recombination of Brainbow in the chosen virus vector. Adeno-associated virus (AAV) vectors that express Brainbow require multiple infections of the same cell to generate a modest number of colors, color spectrum varies with distance from the injection site, and this system appears difficult to use for neuroanatomical applications (Cai et al., 2013). Another approach obtained multiple hues by coinfecting cells with multiple viruses that each express a single, but different, FP: A replication-competent pseudorabies virus (PRV) vector containing a single Brainbow cassette, propagated in the presence of Cre, supports production of progeny virus that express different FPs, and coinfection of the same cell by multiple progeny viruses produces different hues (Kobiler et al., 2010). Further, this approach has been used in transneuronal mapping studies (Card et al., 2011). Nonetheless, this approach labels multiple neurons with the same hue, due to use of a replication-competent vector that labels many neurons. Further, PRV vectors that coexpress membrane-bound and soluble FPs have been used to determine the sequence of connections in a circuit (Boldogkoi et al., 2009). This approach is useful for transneuronal mapping studies, but expression of the FPs is predetermined rather than probabilistic, so the number of hues is limited, and these replication-competent vectors label many neurons with the same hue.

Helper virus-free Herpes Simplex Virus (HSV-1) plasmid (amplicon) vectors (Fraefel et al., 1996; Geller and Breakefield, 1988) have attractive properties for supporting Brainbow expression in small numbers of neurons, thus labeling specific neurons with unique hues. As these vectors are replication-defective, the number of transduced neurons can be experimentally controlled, via the titer of the inoculum. Importantly, the molecular biology of HSV-1 DNA replication and packaging provides a convenient method to produce an array of Brainbow cassettes: HSV-1 DNA replication is biphasic; first, the input DNA molecules are replicated, and, second, rolling circle replication produces concatamers (Roizman and Sears, 1993). With helper virus-free packaging of HSV-1 vectors, an HSV-1 genome-sized (~152 kb) array of concatamers is packaged into HSV-1 particles (Fraefel et al., 1996). For example, using an ~20 kb vector, seven or eight concatamers are packaged into a single HSV-1 particle. Thus, a single infection event could label neurons with a wide range of hues. Moreover, Brainbow recombination might be supported during vector packaging; Cre-mediated recombination can occur during HSV-1 vector replication and packaging (Logvinoff and Epstein, 2001). Thus, as Cre can be present only during packaging, and can be absent from the mammalian brain, the specific Brainbow configuration produced during packaging will be stable thereafter.

Here we describe labeling small numbers of forebrain neurons, and their distant axons, with unique hues, by expressing a Brainbow array from a HSV-1 vector. A Brainbow cassette, containing four axon-targeted FPs, was placed under the control of a glutamatergic neuron subtype-specific promoter (Rasmussen et al., 2007; Zhang and Geller, 2010), and inserted into a HSV-1 vector. Packaging this vector produced arrays of Brainbow, and, following packaging in the presence of Cre, each of the four FP genes was in a position to be expressed, in different Brainbow cassettes. Injection of a vector stock into rat postrhinal (POR) cortex or hippocampus labeled small numbers of neurons in each area with different, and often unique, hues. Further, in an area that receives a large projection from POR cortex, perirhinal (PER) cortex (Agster and Burwell, 2009; Burwell and Amaral, 1998), specific axons were labeled with different hues. Specific axons in PER cortex could be matched to specific neuronal cell bodies in POR cortex, based on hue. As detailed in Section 4, the primary advantage of HSV-Brainbow is the unique capability to label individual neurons with unique hues; in contrast, Brainbow