Methods 52 (2010) 367–374

Contents lists available at ScienceDirect

Methods

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

Review Article

Fluorescent in situ hybridization technique for cell type identification and characterization in the central nervous system

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ARTICLE INFO

Article history:
Available online 14 July 2010

Keywords:
TSA
Tyramide signal amplification
Retrograde tracer
Neuroanatomy
Neocortex
Monkey
Fish

ABSTRACT

Central nervous system consists of a myriad of cell types. In particular, many subtypes of neuronal cells, which are interconnected with each other, form the basis of functional circuits. With the advent of genomic era, there have been systematic efforts to map gene expression profiles by in situ hybridization (ISH) and enhancer-trapping strategy. To make full use of such information, it is important to correlate "cell types" to gene expression. Toward this end, we have developed highly sensitive method of fluorescent dual-probe ISH, which is essential to distinguish two cell types expressing distinct marker genes. Importantly, we were able to combine ISH with retrograde tracing and antibody staining including BrdU staining that enables birthdating. These techniques should prove useful in identifying and characterizing the cell types of the neural tissues. In this article, we describe the methodology of these techniques, taking examples from our analyses of the mammalian cerebral cortex.

1. Introduction

Central nervous system consists of a myriad of cell types, including neurons, glias, endothelial cells, etc. On top of it, each cell type can be further subdivided into many different subtypes [4,27]. Considering that the neuronal circuit is an assembly of various neuronal types, the identification and characterization of each subtype is central to the understanding of the circuit [13]. Recently, systematic efforts to map gene expression in the brain, such as Allen Brain Atlas ([22]; http://www.brain-map.org/), GENSAT ([11]; http://www.gensat.org/index.html) and others (e.g., genepaint.org; http://www.genepaint.org/Frameset.html) have revealed many candidate marker genes for cell type identification. Obviously, certain genes are specifically expressed by particular subsets of neurons. But what are the common features of these neurons? How are they related to the classical neuronal subtypes defined by morphology, electrophysiological and pharmacological properties, antibody staining and connection specificity? What exactly is "cell type" of neurons?

Our laboratory has been trying to identify the unique features of the primate neocortex using molecular biological techniques. Specifically, we have been searching for area- and/or layer-specific genes and using them as probes for comparative ISH analyses [37,41]. What we considered critical in these analyses was the identification of cell types, because, if we want to compare something across species, we need to compare the same thing.

In the cerebral cortex, there are two fundamental cell types, excitatory and inhibitory neurons [23]. These two types can be unambiguously identified by expression of vesicular glutamate transporter 1 (VGluT1) and GABA or GABA synthesizing enzyme GAD, respectively [10,33]. The subtypes of inhibitory neurons can further be classified by expression of several well-known markers [4,7,17]. Because of such specific marker expression, antibody staining has been used extensively to histologically identify these neuronal subtypes. However, some proteins are not localized in the cell body (such as VGluT1) and difficult to be combined with ISH. Furthermore, there are many potentially good marker genes, whose expressions can be detected only by ISH due to lack of good antibodies. It is, therefore, desirable that we can perform dual-probe ISH, in which we can directly compare the mRNA expression of two genes simultaneously at cellular resolution.

Conceptually, dual-probe ISH is similar to immunofluorescent double staining using two antibodies simultaneously. However, the former is often technically more demanding, because the copy number of mRNA molecules could be very low and often requires higher degree of amplification for visualization. The key for success depends on the method of signal amplification. Initially, the detection in ISH was done by using radioactive probes [25]. Then, non-radioactive method using haptens, such as biotin, digoxigenin (DIG), and fluorescein (FITC) for probe labeling became more popular. In a typical method, the hybridized DIG-labeled probe is detected by anti-DIG antibody conjugated with alkaline-phosphatase, which catalytically converts the hybridization signal to

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1046-2023/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2010.07.003

nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) precipitation. By using radioactive and non-radioactive probes for two genes, these methods can be combined for double labeling. Another way for dual-probe ISH is to use different haptens to label two genes and detect them consecutively using the substrates with different colors for alkaline-phosphatase reaction (e.g., see [21]). Although these and other methods of dual-probe ISH have been used successfully for some purposes, most of the methods lacked the resolution and sensitivity comparable to the immunofluorescent double labeling. The only exceptions were those that used tyramide signal amplification (TSA) technique (e.g., [19,20,40]).

TSA is one type of "CARD" or CAtalyzed Reporter Deposition technique [35], in which the horse radish peroxidase (HRP)-conjugated anti-hapten antibody catalyzes the deposition of another hapten, such as biotin, dinitrophenol (DNP), and various fluorescent moieties to its near vicinity. Once the hybridization signal is TSA-amplified, it can be converted to any fluorescent color (see Fig. 1). The fluorescent detection of alkaline-phosphatase activity using HNPP/Fast Red as substrate is also highly sensitive. Thus, at this point, we have several options to visualize the hybridization signals fluorescently. With such advancement at hand, ISH can now be combined with various other histological techniques.

In this paper, we describe the TSA-based dual-probe ISH method, which is useful to visualize diverse cell populations in the cerebral cortex and other brain regions. We also describe the method to combine fluorescent ISH with retrograde tracing, antibody staining and BrdU labeling. The identification of neuronal subtype is often enigmatic because of diversity of neuronal phenotypes. It is also often the case that a particular phonotype is not necessarily an all-or-none property and is a spectrum between 0 and 1. Thus, to identify and characterize neuronal subtypes, it is essential to define properties that are central to the "identity" of each neuron. The ISH-based characterization, combined with various other techniques, has a promise to clarify the complex issue of "cell type". The protocols described here can be found in our past studies [19,38] and are also available at our website (http://www.nibb.ac.jp/brish/indexE.html).

2. Description of method

2.1. Overview

There are many variations of the ISH protocol. The implementation of the fluorescent detection method described herein, however, should be applicable to most protocols. In this paper, we first describe the methodology of free-floating dual-probe ISH, in which free-floating sections are used for hybridization (Section 2.2). We then describe an alternative protocol, in which cryostat sections on a slideglass are used (Section 2.3). These two protocols

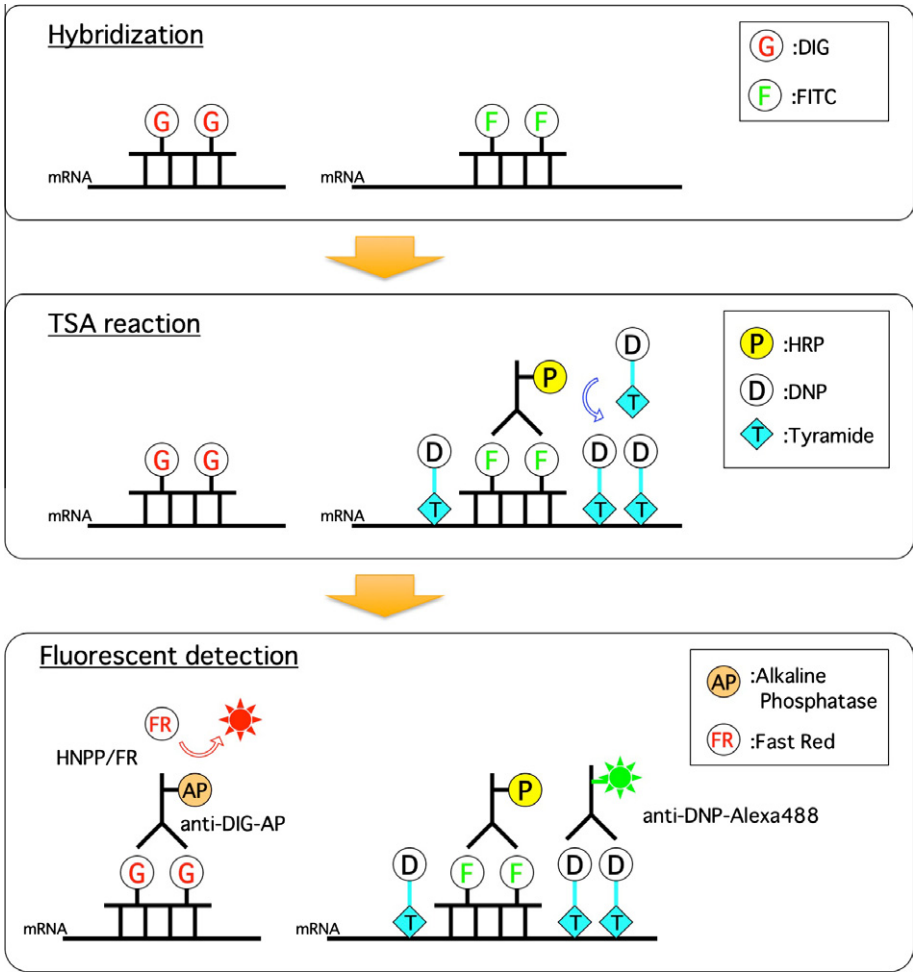

Fig. 1. The scheme for fluorescent double ISH. (Top panel) Digoxigenin (DIG) and FITC (fluorescein)-labeled antisense RNA is hybridized simultaneously to the target mRNAs. (Middle panel) FITC is recognized by anti-FITC antibody conjugated to HRP (horse radish peroxidase), which catalyze TSA reaction, in which the free radical form of DNP–Tyramide reacts to be deposited to the nearby tissue. (Bottom panel) DIG signal is converted to red fluorescence by alkaline-phosphatase activity of the anti-DIG-AP antibody. FITC signal is converted to green fluorescence by anti-DNP antibody conjugated to Alexa 488.