Methods 118–119 (2017) 101–110

Contents lists available at ScienceDirect

Methods

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

Simultaneous detection of the subcellular localization of RNAs and proteins in cultured cells by combined multicolor RNA-FISH and IF

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ARTICLE INFO

Article history:
Received 20 July 2016
Received in revised form 17 September 2016
Accepted 20 September 2016
Available online 21 September 2016

Keywords:
FISH
Immunofluorescence
Antibody
Nucleic acids
GFP
Stress granule

ABSTRACT

Fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) are sensitive techniques used for detecting nucleic acids and proteins in cultured cells. However, these techniques are rarely applied together, and standard protocols are not readily compatible for sequential application on the same specimen. Here, we provide a user-friendly step-by-step protocol to perform multicolor RNA-FISH in combination with IF to simultaneously detect the subcellular localization of distinct RNAs and proteins in cultured cells. We demonstrate the use of our protocol by analyzing changes in the subcellular distribution of RNAs and proteins in cells exposed to a variety of stress conditions.

Contents

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http://dx.doi.org/10.1016/j.ymeth.2016.09.010
1046-2023/

1. Introduction

In eukaryotes, RNA maturation and function are compartmentalized, and RNA transport is a critical step in gene expression. An mRNA molecule starts its life cycle as a nascent transcript transcribed by RNA polymerase II in the nucleus. After capping, splicing, editing, and polyadenylation, the mature mRNA is exported into the cytoplasm. The orderly flow of these processes is coordinated by hundreds of mRNA-binding proteins (RBPs) [1]. In the cytoplasm, the composition of the ribonucleoprotein complexes (RNPs) is extensively remodeled. Specific cytoplasmic RBPs ensure correct mRNA localization, translational regulation, and degradation.

Imaging the intracellular distribution of RNAs and interacting proteins has become of considerable interest and has established links between mRNA localization and protein targeting [2]. The two most widely used techniques to monitor the subcellular localization of nucleic acids and proteins are *in situ* hybridization (ISH) and immunofluorescence (IF), respectively. In 1969, Joseph Gall and Mary Pardue reported the first ISH experiment, in which they hybridized radioactive ribosomal RNA (rRNA) to extrachromosomal ribosomal DNA (rDNA) in *Xenopus tropicalis* oocytes. The RNA/DNA hybrids were visualized in the cytological preparations by tritium autoradiography [3]. ISH is a technique in which labeled single-stranded antisense RNA, DNA, or locked nucleic acid (LNA) probes are hybridized to immobilized RNA or DNA molecules in paraformaldehyde fixed cells or tissues [4,5]. The probes are designed with sequence complementarity to the target nucleic acid to ensure specific base pairing. If the target nucleic acids are double-stranded, they must be denatured prior or during probe hybridization, and fixative nucleobase-modifications interfering with base pairing must be reverted. The nucleic acid probes are typically conjugated to suitable fluorescent dyes or small molecule haptens to allow for subsequent direct or signal-amplification-system-dependent visualization by microscopy, respectively [3,6]. Whereas ISH was originally limited to only abundantly expressed RNAs, improvements in microscopy and fluorescence signal detection, in the stability and quantum yield of fluorophores, and the access to genome-wide transcriptome sequence information impacting specific probe design have allowed for the visualization of single molecules like rare mRNAs and non-coding RNAs [7].

Analogous to sequence-complementarity for RNA-FISH, IF relies on the shape-complementarity of antibody-antigen interactions for detection of proteins of interest. IF was first practiced in the late 1940s by Albert Coons and co-workers who used antibodies conjugated to anthracene or fluorescein isocyanate to specifically detect the presence of the pneumococcal antigen in tissue sections [8,9]. For the visualization of proteins by IF, the specific antibody is either directly conjugated to fluorophores or visualized by the use of a fluorophore-labeled secondary antibody specific to the constant region of the primary antibody. The success of immunofluorescence is dependent on the specificity of the antibody and its compatibility to the immunofluorescence conditions. To circumvent the time-demanding development of highly specific antibodies for every new protein of interest, the protein can be directly expressed as a fusion construct with a short peptide tag, such as FLAG- and HA-tag, for which highly specific monoclonal antibodies have been commercialized for detection [10]. Alternatively, the protein of interest can be fused to a green fluorescent protein (GFP), a widely used marker protein in molecular and cell biology due to its strong intrinsic visible fluorescence. GFP, along with the luminescent protein aequorin, was first discovered and purified from the jellyfish *Aequorea victoria* by Osamu Shimomura in 1962 [11]. After successful cloning of the GFP cDNA by Douglas Prasher [12], Martin Chalfie used the gene to monitor GFP expression and protein localization in *E. coli* and *C. elegans* [13]. Shortly after, Tulle Hazelrigg genetically linked GFP to other proteins and follow the localization and movement of the fusion protein in living cells [14]. The GFP isolated from jellyfish has been engineered to create a variety of blue, cyan, and yellow fluorescent proteins (FPs). Additionally, a variety of FPs from other species have been identified, expanding the available color palette of the FPs to the orange, red and far-red spectral regions [15–17]. Using GFP or its derivatives as a fusion protein has the advantage of directly