Methods 52 (2010) 343–351

Contents lists available at ScienceDirect

Methods

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

Review Article

A practical approach to FRET-based PNA fluorescence *in situ* hybridization

Ana M. Blanco [a], Rubén Artero [b,*]

[a] Sistemas Genómicos S.L., Parque Tecnológico de Valencia, Ronda G. Marconi 6, E-46980 Paterna, Spain
[b] Department of Genetics, University of Valencia, Dr. Moliner, 50, E-46100 Burjassot, Spain

ARTICLE INFO

Article history:
Available online 21 July 2010

Keywords:
Peptide nucleic acid (PNA)
Fluorescence *in situ* hybridization (FISH)
Förster resonance energy transfer (FRET)
mRNA splicing variant
FP-FISH

ABSTRACT

Given the demand for improved methods for detecting and characterizing RNA variants *in situ*, we developed a quantitative method for detecting RNA alternative splicing variants that combines *in situ* hybridization of fluorescently labeled peptide nucleic acid (PNA) probes with confocal microscopy Förster resonance energy transfer (FRET). The use of PNA probes complementary to sequences flanking a given splice junction allows to specifically quantify, within the cell, the RNA isoform generating such splice junction as FRET efficiency measure. The FRET-based PNA fluorescence *in situ* hybridization (FP-FISH) method offers a conceptually new approach for characterizing at the subcellular level not only splice variant isoform structure, location, and dynamics but also potentially a wide variety of close range RNA–RNA interactions. In this paper, we explain the FP-FISH technique workflow for reliable and reproducible results.

1. Introduction

Detection of mRNA splicing variants within cells provides much valuable information on the spatial distribution and compartmentalization of RNA [1]. The use of *in situ* hybridization (ISH) techniques allows to visualize specific RNA molecules at the (sub-) cellular level with spatial and temporal resolution, distance range, and sensitivity, in a broad range of biological applications [2]. Different reports have described the *in situ* detection of specific splicing variants in *Drosophila* embryos [3], striate cortex sections [4], and microdissected eye tissue [5] using DNA oligonucleotide probes. However, the use of DNA probes for RNA-ISH suffers three important limitations. First, the relatively low affinity of DNA hybridization probes for complementary RNA requires that long DNA probes are used (at least 30-mer probes; usually in the 100–1000 nucleotide long range) to increase sensitivity and probability to bind the RNA target. The second disadvantage of DNA probes is that hybridization to the RNA target yields DNA–RNA duplexes that are substrates for RNase H, therefore stimulating target degradation. Finally, the third limitation is saturation of signal making quantification of RNA species very difficult with the use of DNA hybridization probes [6,7]. One way to address all these limitations is to use artificial, high-affinity analogs of DNA, such as peptide nucleic acids (PNAs) and locked nucleic acid (LNA) modified probes. Usually, fluorophores are covalently attached to synthetic DNA analogs for their use as molecular probes to hybridize with a complementary sequence in the RNA of interest [8,9]. PNA has the same hydrogen bonding nucleobases as DNA, but they are attached to an uncharged pseudopeptide backbone. The high affinity of PNAs for RNA, due to the lack of electrostatic repulsion, the ease with which PNAs can be synthesized attached to various fluorescent labels, and the inability of RNase H to recognize PNA–RNA hybrids make this an optimal solution for RNA detection. The efficiency of PNAs as hybridization probes has been demonstrated in fluorescence *in situ* hybridization (FISH) [10]. Cohybridization of two probes labeled with donor and acceptor fluorophores allowed the use of Förster resonance energy transfer (FRET) in the study of structural dynamics [11]. FRET is an easily quantifiable process through which an excited fluorophore (donor) transfers its energy to a nearby light-absorbing molecule (acceptor) [12]. The combination of PNA probes and FRET has been used successfully for the detection of unspliced and spliced versions of RPS14A mRNA by *in vitro* transcription techniques [13], as well as for the detection and quantification of GNAS mutant alleles in fibrous dysplasia/McCune-Albright syndrome [14].

To improve upon existing *in situ* methods, we combined the use of PNAs as hybridization probes and FRET as a widely applied tool to measure distances on the molecular scale in cells, in the novel FRET-based PNA FISH (FP-FISH) technique [15]. The procedure basically involves using two fluorescently labeled PNA probes complementary to sequences flanking a given splice junction. Probes specifically detect mRNA species in which target sequences are close together by engaging appropriate donor/acceptor fluorophore pairs in FRET. The technical improvements associated with FP-FISH method are the following: (a) the high efficiency of PNA probes in FISH by enhancing binding specificity, low background, and unlimited stability of the probe mixture; (b) the use of suitable

* Corresponding author. Fax: +34 96 3543029.
E-mail address: ruben.artero@uv.es (R. Artero).

1046-2023/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2010.07.010

PNA pair fluorophores to increase FRET efficiency in the hybridized cell samples, which allows quantification of the target RNA; and (c) the analysis by FRET-sensitized emission under a software guidance optimizing the image and the measurements of the FRET share being the system easy for the operators to handle. Furthermore, subsequent improvements of the method might allow the use of LNA probes as well as simultaneous detection of two RNA isoforms in a single experiment (Fig. 1). We find also that FP-FISH has two main advantages over other RNA quantification techniques such as Northern blot, RNA protection assays, or RT-PCR: (1) it provides information as for the distribution of a given RNA species within cellular compartments; and (2) detection of FRET serves as a molecular ruler so that closeness between two RNA molecules (or regions within a single RNA molecule) can be assessed, as similarly performed between proteins. The assay might also be adapted to the analysis of RNA–RNA and RNA–protein interactions and is therefore expected to resolve and confirm predicted macromolecular interactions involving RNA, and to find broad application in basic cell biology and molecular pathology research. FP-FISH can allow one to determine if a specific splice region has been removed in a given mRNA, as well as permitting the assessment of close physical proximity of two distinct mRNA molecules.

2. Experimental design

A sound FP-FISH experimental design is the key to achieve effectiveness and reproducibility between the different FRET analyses. It is important to work under tightly controlled and well-defined conditions. First of all, to ensure RNA detection success, it is important to maintain an appropriate sample handling methodology within an RNase-free environment, starting with all the reagents and materials used, continuing through the cell culture, hybridization, and FRET detection. As for experimental procedures and conditions, it is essential to design the correct PNA probes flanking a given splice junction not more than some 25 nt apart as well as to label them with a pair of fluorophores suitable for FRET, such as Cy3-Cy5; Alexa488-Alexa555; Alexa488-Cy3; and FITC-TRITC. The cell culture growth conditions and the FISH protocol used to detect RNA within cells are also key factors to minimize variability and must be selected and assayed depending on the cell type used. Also, different fixation conditions, hybridization, and post-hybridization washes must be optimized to ensure the specificity of the probe–target hybrid formed. Although the analysis by FRET-sensitized emission under software guidance is suitable for standard confocal microscope operators, it is recommended to

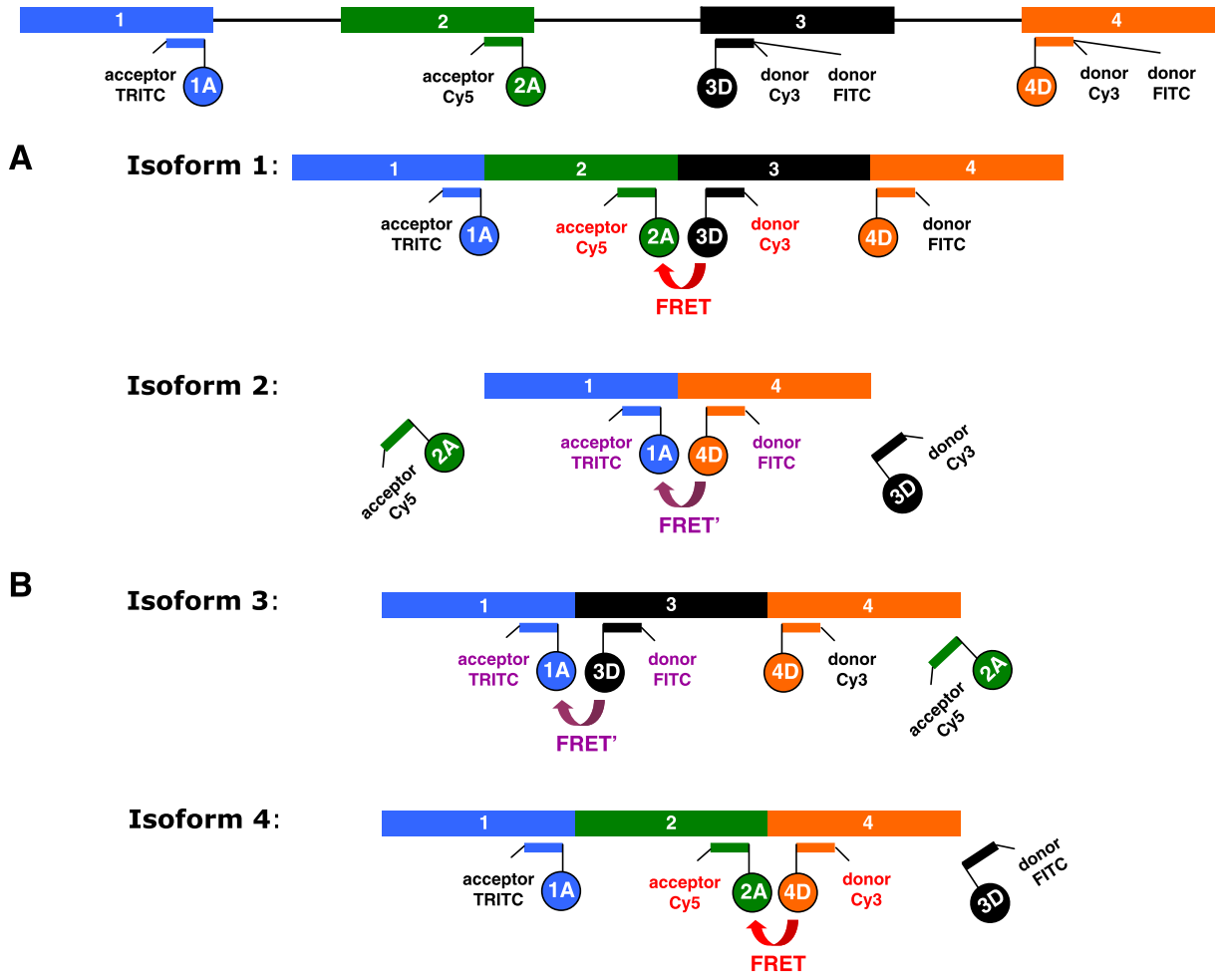

Fig. 1. FRET detection of different splice variants using a combination of PNA probes. The example illustrates a hypothetical situation in which two exons are alternatively spliced giving four possible combinations. Detection of all four splice variants would be theoretically possible employing four donor and two acceptor probes in two independent experiments. (A) First hypothetical experiment with the combination of four different PNAs in the same sample (two acceptors, 1A-TRITC and 2A-Cy5, and two donors, 3D-Cy3 and 4D-FITC). This combination permits the specific detection of both isoforms 1 and 2, because positive FRET could be detected at different excitation and emission wavelengths. (B) Hypothetical second experiment, using different donor PNA probes (3D-FITC and 4D-Cy3). Both isoforms 3 and 4 could be detected as positive FRET with distinct wavelengths. Although PNA probes will hybridize to complementary sequences in the genomic DNA (shown schematically for this example in the top box), because introns separate exonic sequences no FRET is expected for these molecular interactions.