BIOSEARCH® TECHNOLOGIES

Custom Stellaris® FISH Probes

White Paper

Stellaris RNA FISH: *Detection, Localization, and Quantification of mRNA at the Cellular Level*

Abstract

Presented here is a new method for *in situ* RNA detection, the Stellaris® FISH technology, which enables simultaneous detection, localization, and quantification of individual RNA molecules at the cellular level. This technique provides a high degree of specificity and sensitivity, is ideal for applications that analyze transcription site activity, protein-RNA interactions and mRNA translocation events, and holds promise for future diagnostic applications.

Introduction

Methods for *in situ* RNA detection have been in existence for some time, but these techniques have thus far faltered for reasons including specificity, incompatibility with existing research methods, or overly complex preparation methods.

Stellaris FISH technology utilizes a set of simple fluorescent probes which bind along the length of target RNA. The use of multiple probes ensures that positive signal is clearly visible above background without need for amplification, while fluorescence due to stray probes falls below the threshold of detection.

The simple structure and small size of the Stellaris probes makes permeabilization possible without protease treatment. As a result, the procedure is less cumbersome than existing methods and more easily integrates with complementary detection techniques such as fluorescent antibody assays.

Method

The Stellaris FISH protocol is comparatively simple, and consists of four steps, as shown in Figure 1. No exotic reagents are required, and the entire process can be completed in less than a day.

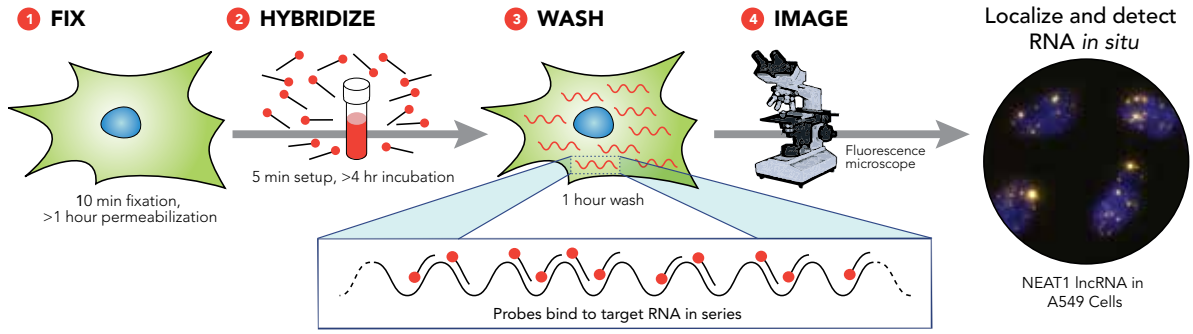

Figure 1: Stellaris assay workflow.

Step 1: Prepare sample

The sample is adhered or sectioned onto a #1 coverglass and permeabilized with 70% EtOH. Slight variations in sample preparation between different organisms and sample types are covered in the online protocols. No protease is required.

Step 2: Hybridize Probes

For most samples, hybridization can be completed in 4 hours at +37 °C in a generic laboratory incubator.

Step 3: Wash Sample

After hybridization, wash buffer with short incubation periods is used to remove excess probes. The total time for this step is 1 – 1.5 hours.

Step 4: Image Sample

At this point, the sample can be imaged using a standard fluorescence microscope.

Sensitivity and Specificity

The large number of probes in a Stellaris FISH assay ensures a high level of sensitivity. While there are various factors such as buffer composition and hybridization temperature that can improve the binding of probes to target RNA, hybridization is, at its root, a stochastic process. As a result, assays which depend on a single probe or a small number of probes will fail to detect a small number of the target RNA. With a larger number of probes, built in redundancy minimizes the risk of a false negative, as enough probes will hybridize to target RNA to allow detection. The effect of the stochastic nature of hybridization can be observed in the small variation in fluorescence among positive spots in a Stellaris FISH assay (figure 2).

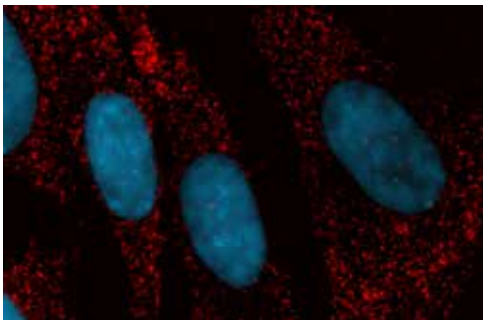

Figure 2: Low levels of variability in fluorescence due to number of hybridized probes.

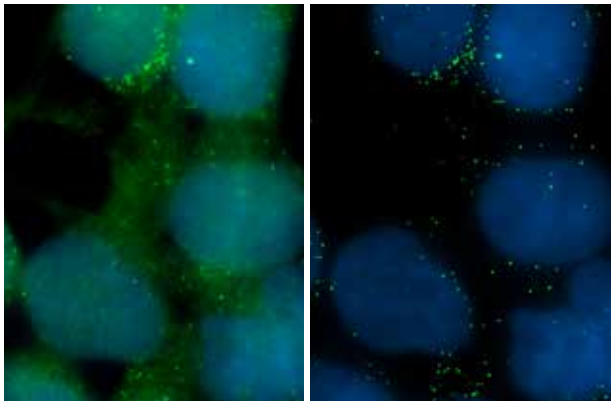

Figure 3: Sample directly after imaging (left), and after background subtraction in ImageJ (right).

Stellaris FISH assays are also highly specific due to the large number of probes. Positive signal is only identified from the combined localized fluorescence of multiple probes. Off-target binding of probes generates weak and diffuse fluorescence, well below the threshold for specifically-targeted mRNA detection. The punctate spots which indicate positive signal clearly stand out from background, and the distinction can be further improved with background subtraction algorithms (figure 3) common in many imaging software packages. In contrast, detection methods which use multiply labeled probes have the potential for a single stray probe to be detectable above background.

The sensitivity and specificity of Stellaris FISH probes has previously been established through comparison with RT-qPCR by Raj et al[1].

Multiplex Capability

Stellaris FISH probes are available with a wide variety of fluorophores, including Biosearch Technologies' CAL Fluor® and Quasar® dyes. Like qPCR, the variety of fluorophores allows the technology to be used in a multiplex assay (figure 4). The dye choices in a multiplex assay are largely determined by the filters available to a researcher, but it is certainly possible to design an assay including a DAPI nuclear stain and three Stellaris FISH probe sets labeled with reporter dyes with minimal spectral overlap.

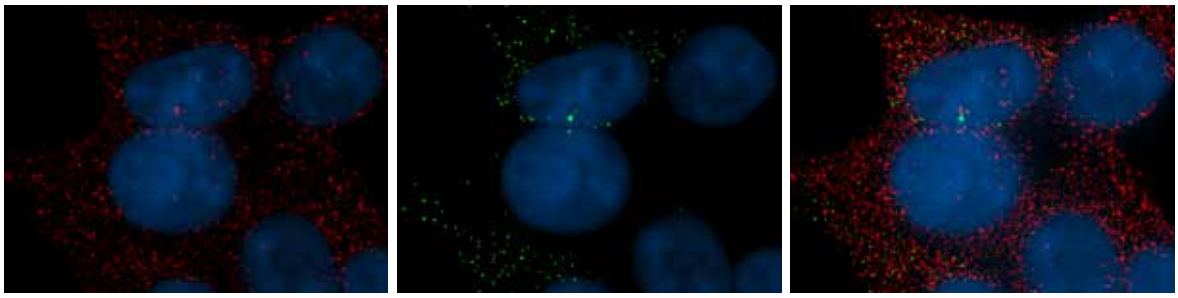

Figure 4:Highly expressed GAPDH (left), Low expression c-myc (middle), Overlay of multiplex (right)

Multiplex assays can be used to identify a correlation in expression levels between multiple genes, run a control probe set of known expression with a target simultaneously, or simply save time over running the assays one at a time.

Combination with Antibody Assays

The use of ethanol instead of protease in the permeabilization step prevents additional proteolysis from the fixation process. As a result, it is possible to combine a Stellaris assay with fluorescent antibody assays (figure 5) to simultaneously image a messenger RNA and the protein for which it codes. This can be a useful tool in identifying mechanisms of translational regulation.

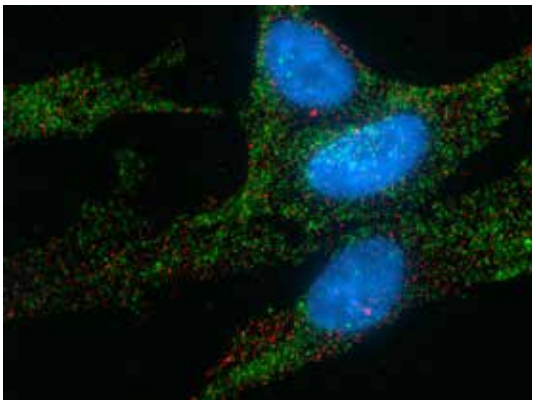

Figure 5: Multiple assay with GAPDH mRNA using Stellaris Probes (green), and Alexa Fluor® 488 antibodies for GAPDH protein (red)

Use in FFPE Samples

Stellaris FISH probes can be used in FFPE samples (figure 6) as well as adherent cells and frozen tissue sections. While freezing is the preferred method of preservation for RNA analysis due to RNA degradation in formalin fixation, in many cases archival FFPE tissue presents the only feasible source of data points. In these cases, Stellaris FISH probes can be used to gain a semi-quantitative analysis of gene expression in archival tissue. Stellaris FISH technology presents an additional advantage over other methods of RNA quantification from archival tissue in that it does not require lysing of the sample and thus keeps tissue morphology intact.

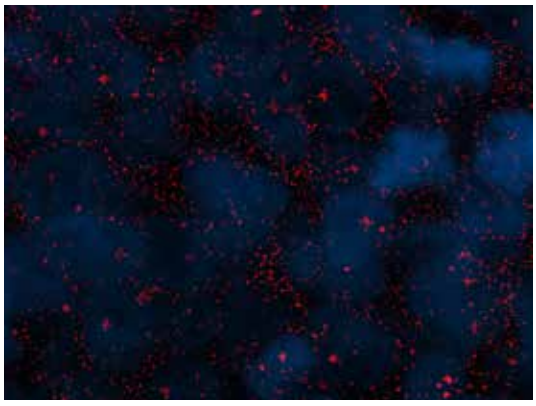

Figure 6: Probes targeting GAPDH mRNA in formalin fixed paraffin embedded (FFPE) tissue.

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