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Stellaris™ fluorescence in situ hybridization (FISH) probes: *a powerful tool for mRNA detection*

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ABSTRACT

The Stellaris™ FISH technology* is a new mRNA detection method that enables simultaneous detection, localization, and quantification of individual RNA molecules at the cellular level. This technique is ideal for applications that analyze transcription site activity, protein-RNA interactions and mRNA translocation events, and holds promise for future diagnostic applications.

INTRODUCTION

Sensitive enough to detect individual molecules of mRNA[1], Stellaris FISH probe sets consist of multiple singly-labeled oligonucleotides (generally 30-48) designed to hybridize along targeted RNA transcripts. Each discrete transcript is observed as a diffraction-limited spot by conventional fluorescence microscopy. The relatively large number of probes used to detect a single mRNA molecule makes this technology less prone to false negatives, since a sufficient fraction of probes will still bind the target mRNA and produce signal despite partial mRNA degradation or obstruction by RNA-binding proteins. Likewise, the potential for false positives is low because the signal is only detectable when a significant number of probes are bound. Off-target binding of probes generates weak and diffuse fluorescence, well below the threshold for specifically-targeted mRNA detection. This *in-situ* technology requires no amplification and provides direct detection of individual molecules of mRNA. Furthermore, Stellaris FISH probes can be labeled with any of a number of different dyes, allowing for simultaneous detection of several mRNA targets. The entire protocol can be carried out in a single day, including cell fixation, probe hybridization, and imaging with a widefield fluorescence microscope.

POSITIVE CONTROLS AND ACTIVE SITES OF RNA TRANSCRIPTION

To supplement Stellaris assays designed for custom targets, positive control probe sets targeting well-expressed mRNAs such as GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were designed and verified. Two distinct Stellaris probe sets targeting the coding sequence of either human (**Fig. 1a**) or mouse (**Fig. 1b**) GAPDH provided very robust signals and revealed mRNAs predominantly localized to the cytoplasmic compartment of the cells, with active sites of transcription appearing in the nucleus as larger and significantly brighter spots due to the accumulation of nascent mRNAs.

In order to further analyze and verify the nuclear spots as active sites of transcription, mRNA transcripts of the HER2 (human epidermal growth factor receptor 2; also known as NEU and ERBB2) gene in SK-BR-3 breast cancer cells were interrogated. In 25% to 30% of all human breast cancers, HER2 is found to be amplified and/or its protein product overexpressed[2], and has emerged as a major biomarker for invasive breast cancer. SK-BR-3 cells contain approximately 11 copies of the HER2 gene[3]. When SK-BR-3 cells were probed with Stellaris probes to HER2, hundreds of cytoplasmic mRNA transcripts were observed. Even more striking, however, were the multiple bright spots of active transcription within the nuclei, often totaling 10 or 11 (**Fig. 1c**). Furthermore, with the addition of the histone deacetylase inhibitor TSA (trichostatin A), HER2 mRNA transcription ceased as evidenced by the disappearance of these bright nuclear spots (**Fig. 1d**). These observations are consistent with reports that TSA inhibits HER2 transcript synthesis and selectively destabilizes HER2 transcripts[4].

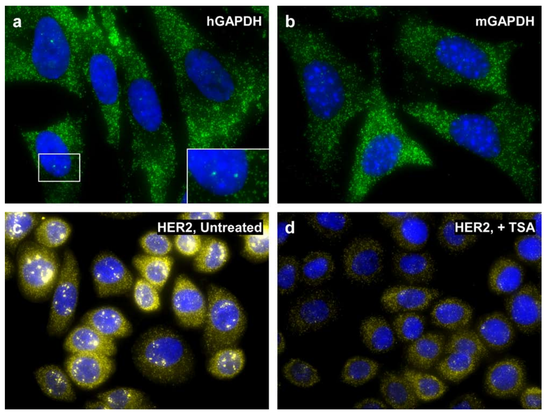

Figure 1 Detection of mRNAs in human and mouse fibroblasts.

Maximum intensity images showing fluorescent spots corresponding to (**a**) GAPDH mRNA in normal human lung fibroblasts (HEL 299), and (**b**) in a mouse embryonic fibroblast cell line (NIH/3T3); the inset shows the sites of active RNA transcription. SK-BR-3 human breast cancer cells probed for HER2 were either (**c**) untreated or (**d**) treated for 6 h with 1 µM TSA.

MULTIPLEXING WITH DIFFERENT PROBE SETS

Two or more distinctly labeled Stellaris FISH probe sets can be combined and used to analyze different mRNA transcripts simultaneously. The potential to multiplex is limited only by the microscope filter set and the equipment's ability to optically separate various fluorochrome signals. As an example, in SK-BR-3 cells, HER2 mRNAs (red) were again visualized using Stellaris FISH probes (**Fig. 2a**), and the same cells were simultaneously probed for ACTB transcripts coding for β-actin (green) (**Fig. 2b**). Ubiquitous and robust expression of both ACTB and GAPDH has made them common standard housekeeping genes. Probing with the HER2 FISH probes produced the anticipated pattern of mRNA localization, including the intense nuclear spots indicative of active transcription. In the cytoplasm, β-actin and HER2 exhibited similar patterns of high expression; however, the number of nuclear spots of active transcription differed substantially as, unlike HER2, ACTB is not amplified in SK-BR-3 cells. In addition, the fluorescent spots of cytoplasmic mRNA for HER2 and β-actin overlapped minimally, further demonstrating specificity of each Stellaris FISH probe set (**Fig. 2d**).

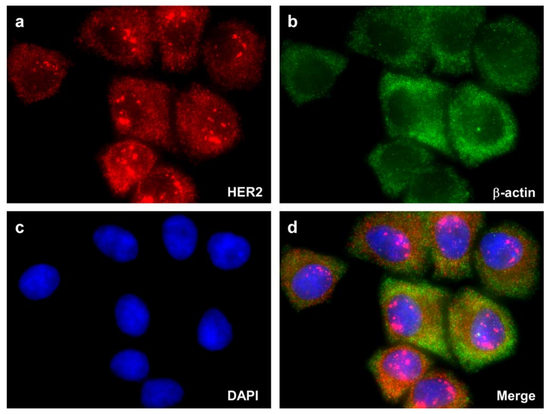

Figure 2 Simultaneous imaging of two different mRNAs in mammalian cells.

(**a**) Fluorescent spots correspond to mRNAs of HER2 (red) in SK-BR-3 cells. (**b**) Fluorescent spots correspond to mRNAs of β-actin in the same cells. (**c**) Nuclei were stained with DAPI. (**d**) A merge of the three different channels.

SENSITIVITY, QUANTIFICATION AND PROBING OF TISSUE SECTIONS

The Stellaris FISH method has been demonstrated above to be quite effective at visualizing mRNA transcripts that are well expressed in most tissues and cells, but it also provides enough sensitivity to detect transcripts at significantly lower levels. When visualized with Stellaris FISH probes, HER2 mRNA was shown to be highly overexpressed in SK-BR-3 cells, averaging 1118 total transcripts (**Fig. 3a and Fig. 3e**). This number far exceeded those observed in two other breast cancer cell lines, MCF-7 (**Fig. 3b**) and MDA-MB-231 (**Fig. 3c**). MCF-7 cells averaged 67 transcripts, and MDA-MB-231 cells averaged 20 transcripts (**Fig. 3e**). This data is entirely consistent with the measurements of protein levels as previously observed by western blot for these three different breast cancer cell lines[5]. In contrast to the semi-quantitative nature of western blotting, the Stellaris FISH technique provides consistent and accurate numbers of total mRNA.

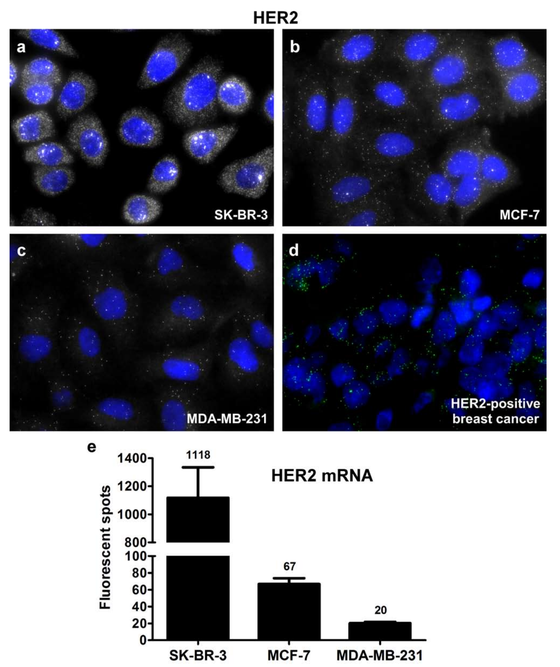

Figure 3 Quantification of mRNAs in various human breast cancer cell lines.

mRNAs of HER2 in (**a**) SK-BR-3, (**b**) MCF-7 and (**c**) MDA-MB-231 cells. (**d**) HER2 mRNA was also detected in human breast cancer tissue. (**e**) SK-BR-3 cells averaged approximately 17-fold more HER2 mRNA than MCF-7 cells and approximately 56-fold more than MDA-MB-231.

Along with immunohistochemistry (IHC) and genomic DNA FISH, Stellaris FISH can thus serve as a valuable companion method to assess the status of HER2 in suspected breast tumors. By using Stellaris FISH we have successfully confirmed overexpression of HER2 mRNA in formalin-fixed paraffin embedded (FFPE) human breast cancer tissue (**Fig. 3d)**. Use of Stellaris FISH Probes to detect HER2 mRNA in routine clinical specimens may eventually help clinicians decide on the appropriate and most effective regimen of patient treatment, whether antibody therapy, hormonal therapy, or chemotherapy.

CONCLUSION

The power and simplicity of the Stellaris FISH method makes it well suited to the detection of mRNA molecules *in situ*; no unusual chemicals, procedures, or equipment are required. More importantly, this technology allows for the measurement of absolute numbers of mRNA molecules, thus providing strong quantitative data, in contrast to traditional western blotting and northern blotting techniques which provide only relative expression levels of protein and RNA, respectively. Not only does the Stellaris method of RNA detection perform successfully in mammalian cells, but it has also been shown to work quite well in human FFPE tissue preparations as well as in other model organisms *e.g., S. cerevisiae, C. elegans, D. melanogaster.*

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