EML4-ALK pre-mRNA and mature mRNA fusion detection using RNA fluorescence *in situ* hybridization (RNA FISH)

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ABSTRACT

Gene fusions that activate otherwise silent signaling enzymes, such as the anaplastic lymphoma receptor tyrosine kinase (ALK), are responsible for a significant number of lung and other cancers. Several drugs that target ALK have found success in the treatment of patients with ALK-gene fusions. DNA fluorescence *in situ* hybridization (FISH) as used for molecular diagnostics for ALK fusions, detects both functionally active and silent (non-transcribed or -translated) fused genes.

We have applied Stellaris® RNA FISH in two approaches to detect EML4-ALK fusion RNAs. In Stellaris RNA FISH pools of directly fluorescently labeled 20-mer oligonucleotides with similar Tm are used for specific and sensitive detection and subcellular localization of the target RNAs.

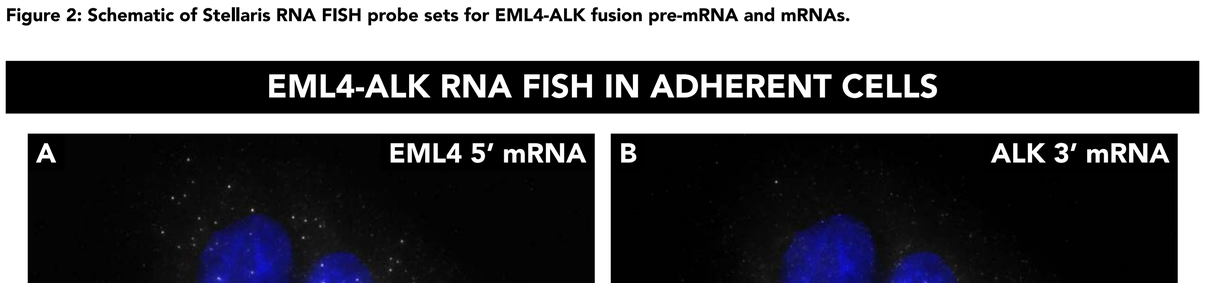
Chromosome 2 inversions (inv2(p21p23)) fuse the promoter and 5′ half of EML4 (active in normal lung tissue) and the 3′ and kinase encoding half of the otherwise silent ALK gene. To detect both mature cytoplasmic mRNAs and nuclear pre-mRNAs from both wild type and fused EML4 and ALK genes, eight RNA FISH probe sets labeled with four different fluorophores were designed against the different 5′ and 3′ segments. We chose the non-small cell lung cancer (NSCLC) adenocarcinoma H2228 cell line that carries one wild type and two mutant chr. 2 with two different inversions (presumed active), for testing.

Quadruplex RNA FISH was carried out on fixed cells with probe sets against each of the different 5′ and 3′ segments of EML4 and ALK mRNAs. Imaging revealed spectrally distinct co-localized signals consistent with wild type cytoplasmic EML4 mRNAs, and with EML4-ALK fusion mRNAs. However, no signal was detected for the ALK 5′ probe set, consistent with the absence of wild type ALK mRNAs. Additionally, larger nuclear foci were detected with the EML4 (5′ and 3′) and the ALK (3′ only) exon probe sets, indicating three active chromosome 2 loci. Active transcription was confirmed for the wild type EML4 gene with probe sets targeting the first and last introns. Signals from the combination of EML4 (first introns) and ALK (last introns) were also found to co-localize on one chromosome, thus verifying the active transcription of one fusion gene. In contrast, at the second mutant chr. 2 locus, only the EML4 (first intron) probe set gave a signal, indicating a non-productive ALK-fusion.

When EML4-ALK probe sets were applied to circulating tumor cells derived from patients with NSCLC, mutant cells were clearly distinguished from wild type cells. Similarly, EML4-ALK fusion mRNAs could be detected in NSCLC tissue samples.

In summary, we have successfully developed and applied Stellaris RNA FISH probe sets to detect mature cytoplasmic and immature nuclear EML4-ALK fusion mRNAs. RNA FISH thus provides a useful tool to identify productive gene fusions in cultured tumor cells, circulating tumor cells and in tissue.

METHODOLOGY



Microscope specifications used for image acquisition:
Nikon Eclipse Ti
Andor Clara CCD camera
Prior Lumen 200 Illumination System
CFI Plan Apo VC 60X Oil

Figure 1. Schematic of Stellaris RNA FISH assay.
For each target, a mix of multiple 20-mer oligonucleotides, each labeled with a single Quasar® 570, Quasar 670, or CAL Fluor® Red 610 fluorophore was designed* and synthesized. All probe sets contained at least 32 oligos. Adherent cells were grown on #1 cover glass and subsequently fixed and permeabilized. Hybridizations were carried out for 4 to 16 hours at 37 °C in 50 µl hybridization solution (10% dextran sulfate, 10% formamide in 2X SSC). Samples were then washed, DAPI stained, and imaged. iceFISH™ was performed using Stellaris probes to target RNA introns. Because introns degrade rapidly after splicing, this allows the measurement of actively transcribing genes.

*Stellaris probe designer and protocols: www.biosearchtech.com/stellaris

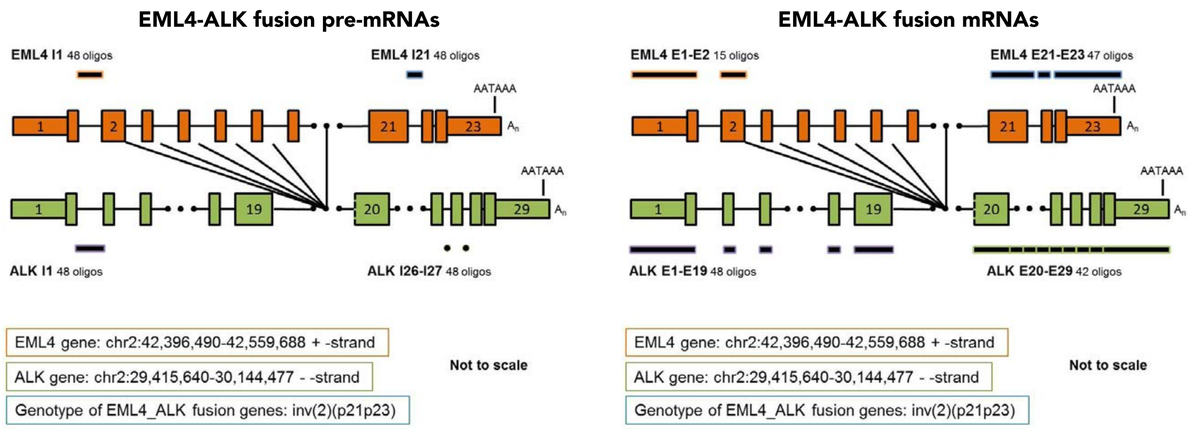


Figure 2: Schematic of Stellaris RNA FISH probe sets for EML4-ALK fusion pre-mRNA and mRNAs.

EML4-ALK RNA FISH IN ADHERENT CELLS

Figure 3: Identification of EML4-ALK mature RNAs in H2228 cells.
(A) A Stellaris probe set labeled with Quasar 570 targets exonic EML4 5′ mRNA in the non-small cell lung cancer cell line, H2228. **(B)** A second probe set labeled with CAL Fluor Red 610 targets exonic ALK 3′ mRNA. **(C)** A third probe set labeled with Quasar 670 targets exonic EML4 3′ mRNA. **(D)** Overlaid images reveal co-localization of EML4 5′ mRNA (green) with EML4 3′ mRNA (red) and EML4 5′ (green) mRNA with ALK 3′ mRNA (blue). Nuclear DAPI counterstaining in blue **(A-C)** and gray **(D)**.

EML4-ALK ICEFISH IN ADHERENT CELLS

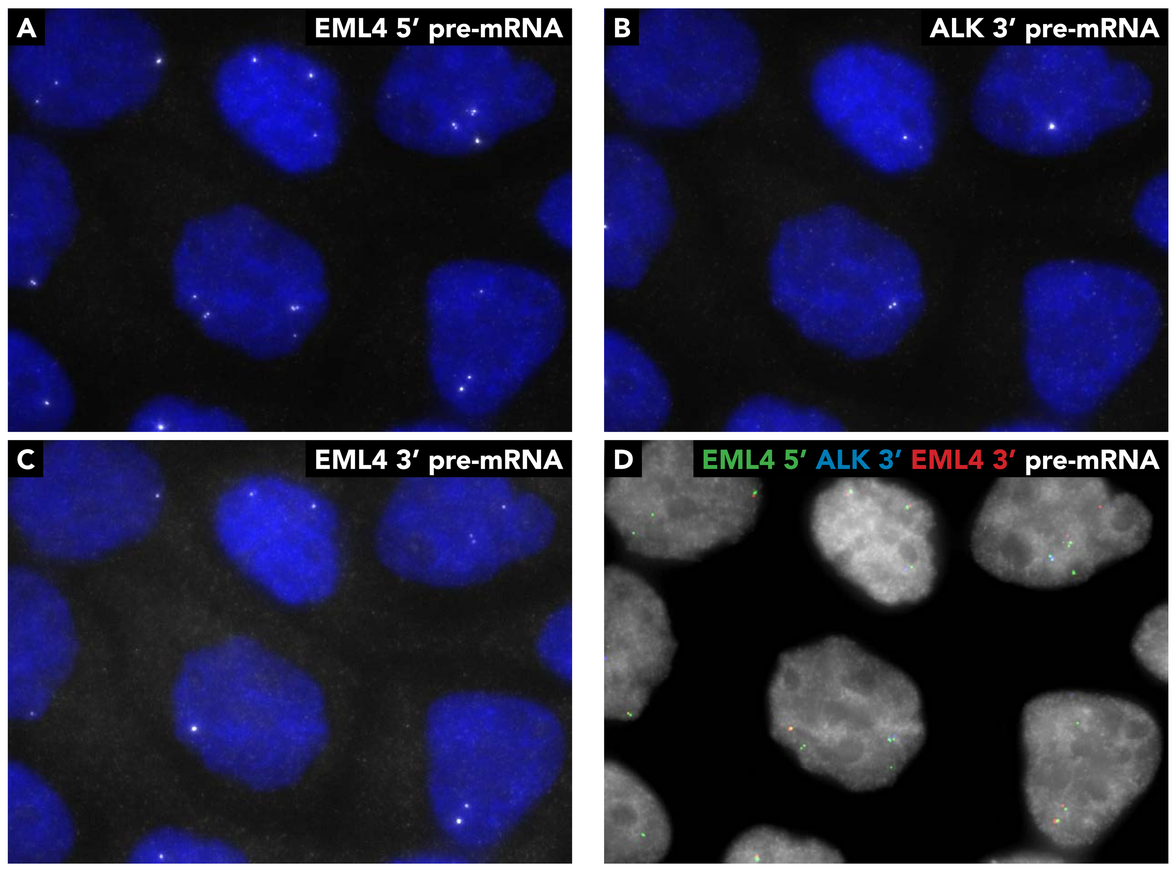


Figure 4: Identification of EML4-ALK nascent RNAs in H2228 cells.
(A) iceFISH was performed using a Stellaris probe set labeled with Quasar 570 targets intronic EML4 5′ RNA. **(B)** A second probe set labeled with Cal Fluor Red 610 targets intronic ALK 3′ RNA. **(C)** A third probe set labeled with Quasar 670 targets intronic EML4 3′ RNA. **(D)** Active transcription for the wild type EML4 gene is confirmed by intronic EML4 5′ RNA (green) co-localizing with the intronic EML4 3′ RNA (red). EML4 5′ RNA was also found to co-localize with ALK 3′ RNA (blue), thus verifying active transcription of one fusion gene. Nuclear DAPI counterstaining in blue **(A-C)** and gray **(D)**.

EML4-ALK RNA FISH IN CIRCULATING TUMOR CELLS

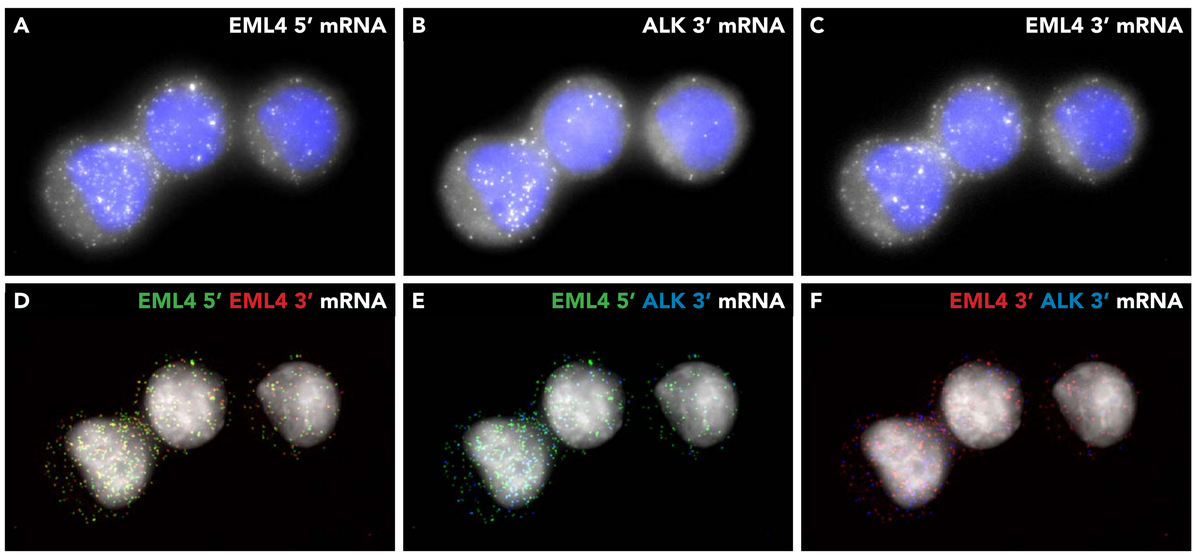


Figure 5: Co-localization of EML4-ALK expression in circulating tumor cells (CTCs).
(A–F) Triplex RNA FISH was performed on CTCs with a Quasar 570 labeled EML4 5′ probe set **(A)**, a Cal Fluor Red 610 labeled ALK 3′ probe set **(B)**, and a Quasar 670 labeled EML4 3′ probe set **(C)**. **(D)** EML4 wild type expression is depicted; EML4 5′ mRNA (green) co-localize with EML4 3′ (red) mRNA. **(E)** EML4-ALK fusion expression is confirmed; ALK 3′ mRNA (blue) co-localize with EML4 5′ mRNA and EML4 3′ mRNA does not co-localize with ALK 3′ mRNA. Nuclear DAPI counterstaining in blue **(A-C)** and gray **(D-F)**.

EML4-ALK RNA FISH IN FFPE TISSUE

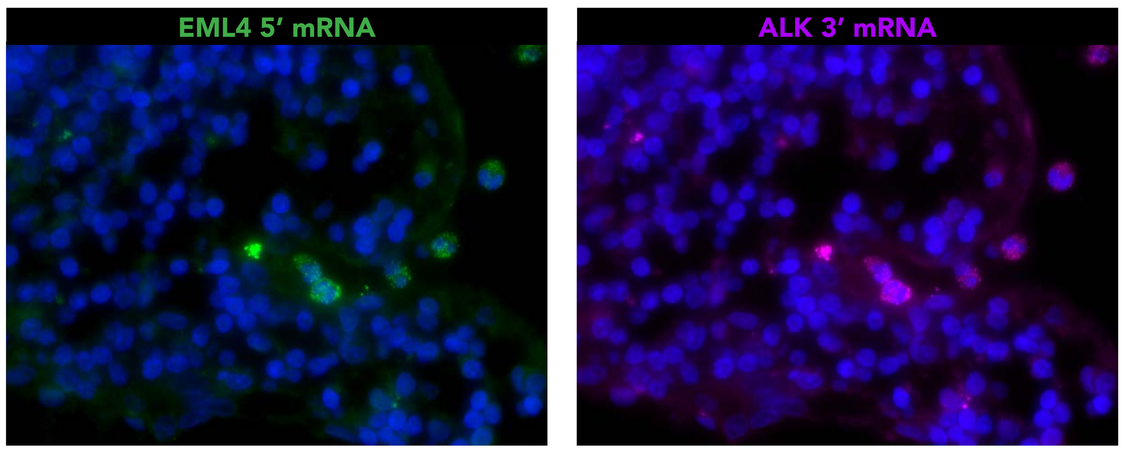


Figure 6: Lung tissue from an EML4-ALK positive lung cancer patient.
The expression of EML4 5′ mRNA (green) and ALK 3′ mRNA (purple) in FFPE lung cancer tissue was assessed by Stellaris RNA FISH. Superimposition of these images reveals co-localized expression.

CONCLUSIONS AND REFERENCES

Stellaris FISH is a powerful method that enables detection, localization, and quantification of RNA at the single cell level. Single molecule RNA FISH (smFISH) provides an accurate method to capture the stochastic behavior of genes, providing further insight into cell-to-cell gene expression variation. By using spectrally distinct fluorescent labels, Stellaris FISH can distinguish different RNA variants from one or multiple genes. Furthermore, because most post-transcriptional processing, including pre-mRNA splicing, occurs co-transcriptionally, RNA FISH targeting the location of introns can be used as a proxy for the encoding gene. Thus, the Stellaris FISH method can serve as a functional proxy for DNA FISH.

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ACKNOWLEDGEMENTS & NOTES

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