Stellaris RNA FISH

Protocol for simultaneous immunofluorescence (IF) + Stellaris RNA FISH in adherent cells

General protocol and storage

Product description

A set of Stellaris™ RNA FISH Probes is comprised of up to 48 singly labeled oligonucleotides designed to selectively bind to targeted transcripts. Stellaris RNA FISH Probes bound to target RNA produce fluorescent signals that permit detection of single RNA molecules as diffraction-limited spots by conventional fluorescence microscopy.

Storage guidelines

Stellaris RNA FISH Probes

Stellaris RNA FISH Probes are shipped dry and can be stored at +2 to +8 °C in this state. Dissolved probe mix should be subjected to a minimum number of freeze-thaw cycles. For daily and short-term use of dissolved probe mix, storage at +2 to +8 °C in the dark for up to a month is recommended. For storage lasting longer than a month, we recommend aliquoting and freezing probes in the dark at -15 to -30 °C.

Stellaris RNA FISH Hybridization Buffer

Stellaris RNA FISH Hybridization Buffer should be stored at +2 to +8 °C for short-term and long-term use.

Stellaris RNA FISH Wash Buffer A and Wash Buffer B

Stellaris RNA FISH Wash Buffers A and B should be stored at room temperature for short-term and long-term use.

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Reagents and equipment

Reagents and consumables:

a) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
b) 37% Formaldehyde Solution
c) 10X Phosphate Buffered Saline (PBS), RNase-free
d) Nuclease-free water
e) Deionised Formamide
f) Ethanol for molecular biology
g) Primary antibody
h) Secondary antibody
i) Stellaris RNA FISH Hybridization Buffer (LGC, Biosearch Technologies Cat# SMF-HB1-10, SMF-HB1-100)
j) Stellaris RNA FISH Wash Buffer A (Biosearch Technologies Cat# SMF-WA1-60, SMF-WA1-600)
k) Stellaris RNA FISH Wash Buffer B (Biosearch Technologies Cat# SMF-WB1-20, SMF-WB1-200)
l) 4′,6-diamidino-2-phenylindole (DAPI)
m) Vectashield® Mounting Medium (Vector Laboratories Cat #H-1000)
n) CoverGrip™ Coverslip Sealant (Biotium Cat# 23005) or clear nail polish
o) 12 or 18 mm round #1 coverglass
p) 12- or 24-well culture plates
q) RNase free consumables such as pipette tips
r) Humidified chamber (or equivalent): 150 mm tissue culture plate; bottom lined evenly with a flat water-saturated paper towel and a single layer of Parafilm® placed on top of the paper towel
s) Superfrost™ Plus Microscope slides
t) 37 °C laboratory oven

Microscope:

a) Wide-field fluorescence microscope (*e.g.*, Nikon Eclipse Ti or equivalent). We provide limited support for confocal applications.
b) A high numerical aperture (>1.3) and 60–100x oil-immersion objective.
c) Strong light source, such as a mercury or metal-halide lamp (newer LED-based light sources may also be sufficient).
d) Filter sets appropriate for the fluorophores.
e) Standard cooled CCD or sCMOS camera, ideally optimised for low-light level imaging rather than speed (13 µm pixel size or less is ideal).

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Preparation of reagents

NOTE: When performing Stellaris RNA FISH, it is imperative to limit RNA degradation. Please ensure that all consumables and reagents are RNase-free. Recipes below are for two set volumes (1X or 10X). Please adjust accordingly.

Reconstituting the dried probe stock:

ShipReady Probe Set (1 nmol):

- A ShipReady probe set can provide up to 80 hybridisations. Re-dissolve the dried oligonucleotide probe blend in 80 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to create a probe stock of 12.5 µM. *Mix well by pipetting up and down,* and then vortex and centrifuge briefly.

DesignReady or Custom Probe Set (5 nmol):

- A DesignReady or custom probe set can provide up to 400 hybridisations. Re-dissolve the dried oligonucleotide probe blend in 400 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to create a probe stock of 12.5 µM. *Mix well by pipetting up and down,* and then vortex and centrifuge briefly.

Fixation Buffer:

Final composition is 3.7% (vol./vol.) formaldehyde in 1X PBS

For a final volume of 10 mL (100 mL), mix:

- 1 mL (10 mL) 37% Formaldehyde solution
- 1 mL (10 mL) 10X Phosphate Buffered Saline (PBS), RNase-free
- 8 mL (80 mL) Nuclease-free water

WARNING! Formaldehyde is a teratogen that is easily absorbed through the skin and should be used in a chemical fume hood. Please consult the appropriate SDS (Safety Data Sheet) prior to use.

Hybridization Buffer (1 mL/10 mL):

Final composition is 10% (vol./vol.) formamide in Hybridization Buffer

Hybridization Buffer should be mixed fresh for each experiment:

- Due to viscosity of the solution, we recommend accounting for a 10% final volume excess in order to have enough Hybridization Buffer for all of your samples.

For a final volume of 1 mL (10 mL), mix:

- 900 µL (9 mL) Stellaris RNA FISH Hybridization Buffer
- 100 µL (1 mL) Deionised Formamide. Mix thoroughly by vortexing and pipetting up and down.

NOTE: Do not freeze Hybridization Buffer.

WARNING! Formamide is a teratogen that is easily absorbed through the skin and should be used in a chemical fume hood. Please consult the appropriate SDS (Safety Data Sheet) prior to use.

WARNING! Be sure to let the formamide warm to room temperature before opening the bottle.

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Wash Buffer A (10 mL/100 mL):

Final composition is 10% (vol./vol.) formamide in 1X Wash Buffer A

Mix and dilute Wash Buffer A fresh for each experiment:

For a final volume of 10 mL (100 mL), mix:

- 2 mL (20 mL) Stellaris RNA FISH 5X Wash Buffer A
- Add 7 mL (70 mL) Nuclease-free water
- Add 1 mL (10 mL) Deionised Formamide. Mix well by vortexing gently.

Wash Buffer B:

Add Nuclease-free water to Wash Buffer B bottle upon first use.

- Add 88 mL (880 mL) of Nuclease-free water to bottle before use. Mix thoroughly.

Nuclear stain for use after hybridisation:

- 4′,6-diamidino-2-phenylindole (DAPI) prepared in Wash Buffer A (see above) at 5 ng/mL. This solution is to be used in Step J below.

Mounting media:

- Vectashield Mounting Medium from Vector Laboratories

NOTE: For best results, samples mounted with Vectashield Mounting Medium should be imaged the same day.

Protocol for Simultaneous IF + FISH in adherent cells

NOTE: This protocol has been adapted for a 12-well plate system. To adapt this protocol for your preferred system, volumes should be adjusted accordingly.

Fixation for Simultaneous IF + FISH in adherent cells

a) Grow cells on 18 mm (or 12 mm) round #1 coverglass in a 12-well (or 24-well) cell culture plate.
b) Aspirate growth medium, and wash with 1 mL of 1X PBS.
c) Add 1 mL of fixation buffer.
d) Incubate at room temperature for 10 minutes.
e) Wash twice with 1 mL of 1X PBS.
f) To permeabilise cells, resuspend cells in 1 mL of 70% ethanol for at least 1 hour at +2 to +8 °C. Cells can be stored at +2 to +8 °C in 70% ethanol up to a week before hybridisation.

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Hybridisation for simultaneous IF + FISH in adherent cells

If frozen before using, warm the reconstituted probe solution to room temperature. Mix well by vortexing, then centrifuge briefly.

To prepare the Hybridization Buffer containing probe, add 1 µL of probe stock solution to 100 µL of Hybridization Buffer, and then vortex and centrifuge (enough for one coverglass). This creates a working probe solution of 125 nM. This solution will be used on steps d and e.

a) Aspirate the 70% ethanol off the coverglass containing adherent cells within the 12-well plate.
b) Add 1 mL of Wash Buffer A (see recipe above), and incubate at room temperature for 2–5 minutes.
c) Assemble humidified chamber: 150 mm tissue culture plate; bottom lined evenly with a flat water-saturated paper towel and a single layer of Parafilm placed on top of the paper towel. This chamber will help prevent evaporation of the probe solution from under the coverglass.
d) Within the humidified chamber, dispense 100 µL of the Hybridization Buffer containing probe plus appropriately diluted primary antibody, onto the Parafilm.
e) Gently transfer the coverglass, cells side down, onto the 100 µL drop of Hybridization Buffer containing probe and primary antibody.
f) Cover the humidified chamber with the tissue culture lid, and seal with Parafilm.
g) Incubate in the dark at 37 °C for at least 4 hours (Incubation can be continued up to 16 hours).
h) Gently transfer the coverglass, cells side up, to a fresh 12-well plate containing 1 mL of Wash Buffer A plus appropriately diluted secondary antibody.
i) Incubate in the dark at 37 °C for 30 minutes.
j) Aspirate Wash Buffer A, and then add 1 mL of DAPI nuclear stain (Wash Buffer A consisting of 5 ng/mL DAPI) plus appropriately diluted secondary antibody.
k) Incubate in the dark at 37 °C for 30 minutes.
l) Aspirate the DAPI staining buffer, and then add 1 mL of Wash Buffer B. Incubate at room temperature for 2-5 minutes.
m) Add a small drop (5-15 µL) of Vectashield Mounting Medium onto a microscope slide, and mount coverglass onto the slide, cells side down.
n) Gently wick away excess anti-fade from the perimeter of the coverglass.
o) Seal the coverglass perimeter with CoverGrip (or clear nail polish), and allow to dry.
p) If necessary, gently wipe away any dried salt off the coverglass using water.

NOTE: When processing multiple samples, steps c-h also can be carried out in the 12 or 24-well plate or in chambered cover slips in which the cells were grown, keeping the wells properly sealed. Volumes should be adjusted accordingly.

Proceed to imaging.

References

1. Orjalo, A.V. Jr., and Johansson, H.E. Stellaris® RNA fluorescence in situ hybridisation for the simultaneous detection of immature and mature long noncoding RNAs in adherent cells. Methods Mol. Biol. 2016; 1402, 119-134. doi: 10.1007/978-1-4939-3378-5_10
2. Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Methods 2008; 5, 877-879. doi: 10.1038/nmeth.1253
3. Femino, A.M., Fay, F.S., Fogarty, K., and Singer, R.H. Visualisation of single RNA transcripts *in situ*. Science 1998; 280, 585-590. doi: 10.1126/science.280.5363.585

Guidelines citing the use of Stellaris RNA FISH Probes and methods in scientific publications

Please acknowledge the use of Stellaris RNA FISH Probes and/or protocols in the experimental **Materials and Methods** or **Methods** section of your manuscript. Refer to the following examples as guidelines for proper citation of the Stellaris RNA FISH Probe sets and/or protocols:

Citing catalogued probe sets:

"Stellaris™ RNA FISH Probes recognising <catalogued gene set name> and labelled with Quasar™ 570 dye (Catalog #, LGC, Biosearch Technologies, Petaluma, CA) were hybridised to <samples>, following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing Custom Probe sets designed with the Stellaris FISH Probe Designer:

"Custom Stellaris™ RNA FISH Probes were designed against <your RNA of interest (include accession number and nucleotides covered if relevant)> by utilising the Stellaris RNA FISH Probe Designer (LGC, Biosearch Technologies, Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (version #). The <samples> were hybridised with the <your RNA of interest> Stellaris RNA FISH Probe set labelled with <your dye of choice> (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing Custom Probe sets utilising previously published sequences:

"Custom Stellaris™ RNA FISH Probes recognising <your RNA of interest (include accession number and nucleotides covered if relevant)> and labelled with <your dye of choice>, were purchased from LGC, Biosearch Technologies (Petaluma, CA). Probe set sequences utilised in the experiments have been previously described <cite published manuscript>. The <samples> were hybridised with the <your RNA of interest> Stellaris RNA FISH Probe set, following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

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Citing 3′ Amine Oligos in plates used for Stellaris RNA FISH designed with the Stellaris FISH Probe Designer:

"Custom 3′ amine oligos in plates were designed against <your RNA of interest (include accession number and nucleotides covered if relevant)> by utilising the Stellaris™ RNA FISH Probe Designer (LGC, Biosearch Technologies, Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (version #). Probes were labelled with <your dye of choice> using <insert your labellling protocol or citation of previously published labelling protocol>. The <samples> were hybridised with the <your RNA of interest> oligonucleotides (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing 3′ Amine Oligos in plates used for Stellaris RNA FISH using previously published sequences:

"Custom 3′ amine oligos in plates recognising <your RNA of interest (include accession number and nucleotides covered if relevant)> were purchased from LGC, Biosearch Technologies (Petaluma, CA). Probe set sequences utilised in the experiments have been previously described <cite published manuscript>. Probes were labelled with <your dye of choice> using <insert your labelling protocol or citation of previously published labelling protocol>. The <samples> were hybridised with the <your RNA of interest> oligonucleotides (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Technical support

If you require additional information or technical assistance, please feel free to email our Technical Support Team at: techsupport@lgcgroup.com.

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