Oct 13, 2024 Version 1

IFA and FISH combined protocol V.1

DOI
https://dx.doi.org/10.17504/protocols.io.j8nlk8or6l5r/v1

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Protocol Citation: Carolina Lopez 2024. IFA and FISH combined protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlk8or6l5r/v1

Protocol status: Working
We use this protocol and it's working

Created: May 24, 2024

Last Modified: August 12, 2025

Protocol Integer ID: 100597

Keywords: preps with both rna fish, rna fish, rna, immunofluorescence, staining prep, ifa, fish

Abstract

This protocol describes the procedures for staining preps with both RNA FISH and immunofluorescence

Materials

REAGENT	SOURCE	IDENTIFIER
Formamide (Deionized)	Thermo Fisher Scientific	Cat#AM9342
RNAseZAP	Sigma Aldrich	Cat # R2020-250ML
ProLong Diamond Antifade mountant	Thermo Fisher Scientific	Cat #P36961
20xSSC	Thermo Fisher Scientific	Cat#AM9770
PBS 1x	Thermo Fisher Scientific	Cat#10010-023
Ultrapure Nuclease Free Water	Thermo Fisher Scientific	Cat#10977-015
38% Formaldehyde	Thermo Fisher Scientific	Cat# F79-1
200 Proof Ethanol (1 pint)	Decon laboratory	Cat# 2716
RNA-FISH probes	Biosearch Technologies	Probe sequence in excel file below
BSA	Sigma	A3059-50G
RNAseOut	Thermo Fisher	10777019

Materials:

1. Nuclease Free water (NF H_2O)
2. Formamide
3. 20X SSC and 2X SSC
5. PBS
9. 70% EtOH
10. Probe: 1 µL/reaction

* Probe prep: Stock ([M] 12.5 micromolar (µM) diluted in TE buffer)

SeV probes: 1:10 working solution (1.25uM) diluted in H_2O, 5 µL stock + 45 µL UltraPure H_2O, in vivo only

SeV probes: 1:100 working solution (125nM) diluted in H_2O, 5 µL 1:10 + 45 µL UltraPure H_2O, in vitro only

RSV probes: 1:50 working solution (250nM) diluted in H_2O, 10 µL 1:10 + 45 µL UltraPure H_2O, in vitro only

Solutions:

1. Fixation solution (4% FA):

(prepare fresh) 5 mL 37% formaldehyde (100% formalin),
45 mL PBS 1X

2. Wash buffer:
(prepare fresh)
5 mL 20x SSC
5 mL formamide
40 mL NF H_2O

3. 70% EtOH:
70 mL 100% EtOH,
30 mL NF H_2O

4. Hybridization buffer: 1 g dextran sulfate in 7 mL NF H_2O (mix by rotation, take half an hour to dissolve).
1 mL formamide
1 mL 20x SSC
Volume up to 10 mL
* Store at -20 °C in 500 µL aliquots; good for years.

5. Mounting media: ProLong Diamond Anti-fade Mountant (Cat #P36961)

6. For GLOX anti-fade:

Anti-fade buffer:
(per slide)
850 µL Nuclease-Free H_2O
100 µL 20x SSC
40 µL 10% w/v glucose
10 µL Tris-HCl (pH 8)

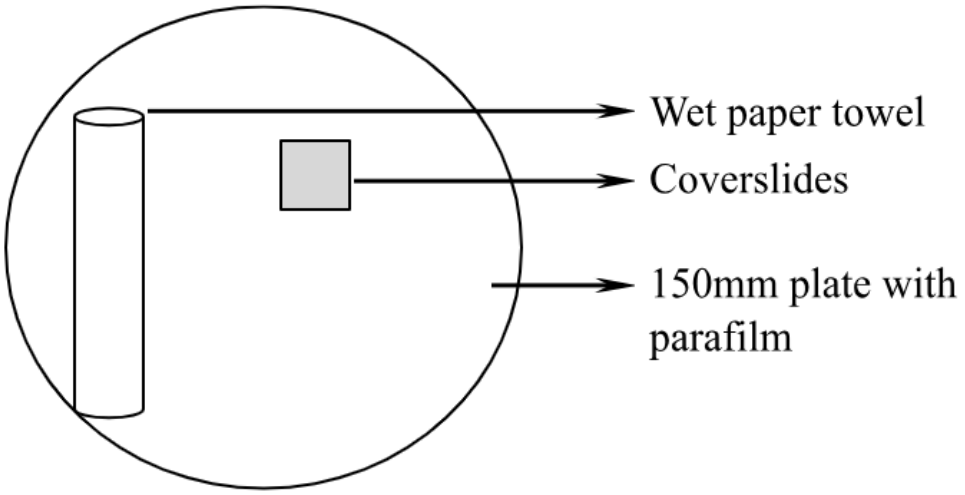
Anti-fade solution:
100 µL Anti-fade buffer
1 µL glucose oxidase
1 µL catalase

Combine FISH with IFA

1 When IFA and FISH are to be combined, we prefer to perform IFA (under RNAase-free conditions) prior to FISH, because the formamide treatment during the FISH procedure is sometimes incompatible with preservation of the epitopes detected by some antibodies.

Equipment:

2 Hybridization chamber:



Protocol:

3 **Before starting, make sure to clean everything with RNaseZAP (Cat # R2020-250ML) and ethanol (i.e. gloves, pipettes, surfaces, etc) and always use filter tips.** 6h 50m

A. IFA:
1. Briefly rinse cells cultured on coverslips in sterile PBS.
2. Fix in filter-sterilized 4% formaldehyde for 00:10:00 at RT. (can move from TC hood to bench after this step)
3. Wash 3X in 8PBS.
4. Permeabilize with ice-cold 70% Ethanol for 01:00:00 (can leave O/N at 4 °C for up to a week)
5. Wash 3X in PBS.

6. Incubate with primary antibody diluted in 1% BSA in sterile PBS (2 µL RNAase OUT per 500 µL) for 00:45:00 at RT in a dark and humid chamber.
7. Wash 3X in PBS.
8. Incubate with secondary antibody (diluted in the same solution as in step 6) for 00:40:00 at room temperature in a dark and humid chamber.
9. Wash at least 3X in PBS.
10. Post-fix in freshly made 4% formaldehyde for 00:10:00 at RT.
11. Wash cells with 2X SSC and then with wash buffer, 00:05:00 each.

B. FISH

--If frozen before using, thaw the reconstituted probe solution to room temperature. Mix well by vortexing, then centrifuge briefly.
--To prepare the hybridization solution, add 1 µL of probe working solution to 50 µL of hybridization buffer, and then vortex and centrifuge. *similar to anitbody concentration, probe concentration should be optimized for each probe set.
1. Aspirate the 70% ethanol off the coverglass.
2. Add 1 mL of wash buffer, and incubate at RT for 2-5 minutes.
3. Prepare hybridization mix and assemble humidified hybridization chamber: see equipment
4. Add 50 µL of the hybridization buffer containing probe onto the parafilm.
5. Gently transfer the coverglass, cells side down, onto the hybridization solution. Cover the humidified chamber with the tissue culture lid. Incubate in the dark at 37 °C for at least 04:00:00, better O/N.

Wash

4 1h

1) Transfer the cover glass, cells side up, to a fresh plate containing 1 mL of wash buffer, immediately.
Incubate in the dark at 37 °C for 00:30:00 .
2) Wash with 1 mL wash buffer consisting of 5 ng/mL DAPI to counterstain the nuclei.
Incubate in the dark at 37 °C for 00:30:00 .
3) Wash with 1 mL of 2X SSC. Incubate at RT for 2-5 min.
4) Mounting slides: Put a drop of ProLong Diamond Antifade (Cat #P36961) on to slide, dry off slide using aspirator and flip cell side down on to mounting media, make sure

there are no bubbles. Mounting media needs to “cure” to correct refractive index O/N at RT (in dark), then can be moved to slidebox/proceed to imaging.

- Alternatively, GLOX anti-fade may be used if necessary/want to image right away. In this case, seal the coverglass perimeter with rubber cement, and allow to dry. Store slides on ice. If necessary, gently wipe away any dried salt off the coverglass with water. Proceed directly to imaging.

Lopez Lab Specific Section:

5 Probe preparation for hybridization:
When adding 1 µL of each probe in 50 µL of hybridization buffer, the final working probe solution should be 125 nM for SeV probes and 250 nM for RSV probes. Adjust depending on the probe set, virus and optimized dilution tested.
* It is always recommended to have immunofluorescence slides staining for viral protein as controls for efficient infection.

Protocol references

Updated by: Lavinia Gonzalez 03/31/23

Protocol adapted from BioreserachTech. For the original protocol:
https://www.biosearchtech.com/assets/bti_stellaris_protocol_adherent_cell.pdf