This protocol is provided as a guideline and may need to be optimized by the researcher

DNA fluorescent *in situ* (DNA FISH) Hybridization of Fixed Cultured Human Cells

Cells are grown to confluency on circular 18-20 mm #.15 coverglass.
For all rinses coverglasses are placed in a 12 well multiwell plate, cell side up.
Avoid drying of cells between steps.
All rinses are done with gentle rocking/agitation.

For reconstitution of shipped myTags *in situ* hybridization probes see:
Arbor Biosciences/myTags Protocols-*"Reconstitution of myTags ISH Probes"*
Aliquot probes into working stock volumes to avoided repeated freeze-thaw cycles.

Fixation:

1. Remove media, rinse cells 3X with fresh 1X PBS (PBS), pH 7.4 (Thermo Fisher cat# 10010049).
2. Fix cells for 10 minutes at room temp (RT) in 4% paraformaldehyde (in PBS).
3. Remove fix and rinse cells at RT, 3X 5 minutes each in PBS.
4. Permeabilize cells at RT for 5 minutes with 0.5% triton X-100 in PBS.
5. Rinse cells in 1X PBS. At this point cells can be stored at 4ºC for one day. Otherwise continue with Day 1 Hybridization.

Day 1 - Hybridization:

1. Preheat appropriate vol. of hybridization buffer (HYB) to 37°C.
2. Incubate cells at RT for 30 mins. with 20% glycerol in PBS.
3. Flash freeze the cells by placing the coverslips in liquid nitrogen for 30 sec then thaw for 1 min.
4. Incubate the cells at RT for 20 mins in the glycerol/PBS solution.
5. Repeat flash freeze in liquid nitrogen (step 2).
6. Incubate cells at RT for 5 mins. with 0.1 N HCl.
7. Rinse cells at RT, 3X 1 min. each with 2XSSC.
8. Pre-hybridize cells at 37°C for 30 mins in 500 µl of HYB with no probes.
9. Dilute the myTags probe stock in the HYB at the desired concentration (recommended starting concentration is 10 pmol/hybridization reaction). Use 100µl HYB buffer + probe per reaction (coverslip).
10. Heat the probe+HYB solution for 5 min. at 70°C for 5 mins and chill on ice until use.
11. Remove the pre-hyb solution.
12. Add probe+HYB to HybriSlip™ (Grace Bio-Labs) or parafilm "slip". Invert coverslip with cells down onto probe+HYB mix.
13. Denature in humid environment at 78ºC for 5 mins.
14. Hybridize the cells overnight in humid environment at 37°C.

Day 2 - Post Hybridization:

1. Preheat appropriate vol. of hybridization buffer (HYB) to 37°C.
2. Preheat appropriate vol. of probe wash buffer to 37°C.
3. Remove probe+HYB by washing at 37°C for 2X 30 mins each with 500 µl of preheated probe wash buffer.
4. Wash at RT for 2X 5 mins. each with 5X SSCT.
5. Wash with PBS for 5 minutes at RT.
    a. Add additional washes if excessive background is detected.
6. Stain with appropriate nuclear stain per SOP (recommend-Hoechst 33342 Solution, Thermo Fisher #62249), rinse and coverslip with antifade media (recommend-ProLong Diamond, Thermo Fisher # P36965 or # P36961).

REAGENTS

Probe Hybridization Buffer:

30% Formamide
5 X SSC
9 mM Citric Acid, pH 6.0
0.1 % Tween 20
0.1 µg/µl Human Cot1 DNA
1x Denhardt's Solution
10% Dextran Sulfate
0.4 mg/ml BSA

Make fresh just before use-combine:
3 ml Formamide
2.5 ml 20X SSC
90 µl 1M Citric Acid, pH 6.0
100 µl 10% Tween 20
200 µl 50X Denhardt's Solution
4 ml 50% Dextran Sulfate
50 µl BSA 20 mg/ml (Fisher cat# PR-W3841).
Bring to volume of 10 ml with molecular grade H_2O.

Probe wash buffer:

30% Formamide
5 X SSC
9mM Citric Acid, pH 6.0
0.1% Tween
Stored at -20°C, expiration- 7 days

Combine:
3 ml Formamide
2.5 ml of 20X SSC
90 µl of 1 M Citric Acid, pH 6.0
100 µl of 10% Tween 20
Bring to volume of 10 ml with molecular grade H_20.

5 X SSCT (5X SSC with 0.1 %Tween 20):

Combine:
10 ml 20X SSC
400 µl10% Tween 20
Bring to volume of 40 ml with DEPC H_20
Stored at 4°C, expiration- 1 month.

0.5% Triton X-100:

0.5 ml Triton X-100 in 100 ml 1X PBS

20% glycerol:

20 ml of glycerol in 80 ml 1X PBS

0.1 N HCl:

500 ml, Fisher Scientific cat# S25354

Source;
Zheng et al. (2019) Multiplex chromatin interactions with single-molecule precision. Nature 566(7745):558-562