Protocol PNA FISH (Fluorescence In Situ Hybridization)

FISH (fluorescence in situ hybridization) is a cytogenetic technique to detect and localize the presence or absence of specific DNA sequences on chromosomes.

Because of its high affinity and specificity to target DNA, PNA (peptide nucleic acid) probes are ideal tools for FISH. The benefits are specific target binding, short hybridization time, low background, good reproducibility, and superior stability of the reagent. PNA FISH probes are also efficient at penetrating the tissues thanks to the small size, and no need for denaturation of the probe itself since it is a short single-stranded oligonucleotide (PNA Bio).

Chemicals & materials

Chemicals

Chemicals	Provider (article number)
PNA probes (Alexa488-labeled centromere probe & Cy3-labeled C-rich telomere probe)	Eurogentec (PN-CN060-005 & PN-TC050-005)
Formamide	Roth (P040.1)
Ultrapure TRIS	PanReac Applichem (A1086,1000)
Blocking reagent	Roche (11096176001)
ROTI stock 20X SSC	Roth (1054.1)
Tween-20	Roth (9127.1)
Pure ethanol	Roth (5054.1)
DAPI	Roth (6335.1)
Nuclease-free water	Qiagen (129117)
ROTI stock 10X PBS	Roth (1058.1)
VECTASHIELD® Antifade Mounting Medium	Biozol (VEC-H-1000)
DNA AWAY	Carl Roth (X996.1)
RNA AWAY	Carl Roth
DPBS without Ca and Mg	PAN Biotech (P04-361000)
RPMI 1640 medium	Gibco (11875093)
Trypsin inhibitor	Roth (2949.1)
Melanocyte Growth Medium 3	PromoCell (C-24310)
Keratinocyte Growth Medium 2	PromoCell (C-20011)

Materials

Material	Provider
T25 flasks	TPP
Serological pipette (5, 10 & 25 ml)	Sarstedt
PIPETBOY acu 2 pipette controller	Integra
7.5 ml pasteur pipette	
Microliter pipette (10, 200, & 1000 µl)	Eppendorf
RNAse-free pipette tips	Sarstedt
RNAse-free SafeSeal reaction tubes	Sarstedt
Microscope slide	ThermoFischer
Metal box	-
Centrifuge	Eppendorf
Centrifuge tubes (15 & 50 ml)	Corning

Plastic strip	-
Incubator	Binder
ThermoCycler	Eppendorf
Staining cuvette	-
Confocal microscope	Zeiss Observer Z1 and LSM 710

Reagents

0.5 mg/ml DAPI stock solution

25 mg DAPI
+ 5 ml 2X SSC
➔ Store at -20°C

1X PBS

10% 10X ROTI stock 10X PBS (100 ml)
90% nuclease-free water (900 ml)
➔ Store at room temperature

2X SSC

10% 20X ROTI stock 20X SSC (10 ml)
90% nuclease-free water (90 ml)
➔ Store at room temperature

50 µM (250 µl/mg) PNA probes

5 nmol PNA probe
+ 100 µl formamide
➔ Store at -20°C

50 mM TRIS/1.25% (w/v) blocking reagent stock

50 mM Ultrapure TRIS (0.242 g)
1.25% blocking reagent (0.5 g)
Nuclease-free water (40 ml)
➔ Store at +4°C

70% (v/v) Ethanol

70% pure ethanol (140 ml)
30% nuclease-free water (60 ml)
➔ Prepare fresh

85% (v/v) Ethanol

85% pure ethanol (170 ml)
15% nuclease-free water (30 ml)
➔ Prepare fresh

DAPI solution

1 part 0.5 mg/ml DAPI stock solution (1 µl)
749 parts 2X SSC (749 µl)
➔ Prepare fresh

Fixative

1 part acetic acid (50 ml)
3 parts methanol (150 ml)

➔ Store at -20°C

Hybridization buffer

40% 50 mM TRIS/1.25% blocking reagent stock (400 µl)
60% formamide (600 µl)

➔ Prepare fresh

Wash solution

10% 20X SSC (100 ml)
0.1% Tween-20 (1 ml)
89.9% Nuclease-free water (899 ml)

➔ Store at room temperature

Cell preparation

- On the first day, seed the cells in T25 flasks with defined cell density in growth medium.
- Pre-culture the cells for 24 h.

Treatment

- On the second day, change the medium with 8 ml growth medium.
- Expose the cells to electromagnetic fields for defined exposure time.
- Incubate the cells in the incubator for further 1.5 cell cycle to allow micronuclei to form.
- If needed, change the medium after 48 h of seeding.

Fixation

- Wash the cells twice with 3 ml DPBS without Ca^{+} and Mg^{+}.
- Detach the cells using 3 ml trypsin for 5 minutes at 37°C (0.05% trypsin for cell lines and 0.04% trypsin for primary cells).
- Stop the detachment process using 3 ml serum-containing medium (cell lines) or 50 µl trypsin inhibitor in 3 ml cell medium (primary cells).
- Resuspend the cell suspension.
- Take 100 µl aliquot of cell suspension for cell counting.
- Centrifuge cells at room temperature, 1000 rpm for 5 minutes.
- Remove the supernatant.
- Resuspend the pellet with 200 µl (primary cells) or 300 µl PBS (cell lines).
- Pre-heat a heating plate to 65°C.
- Drip the cell suspension using Pasteur pipette from 10 cm above onto cleaned and frozen glass microscope slides on an ice plate.
- Dry the slides on the heating plate.
- Fix the cells in a staining cuvette with fixative for 5 minutes in the exhaust hood.
- Dry the slides in the exhaust hood for 15 minutes.
- Store the slides at -20°C until further use.

Hybridization

- Dehydrate the prepared slides by incubating the slides in 70%, 85%, 100% (v/v) ethanol for 2 minutes each.
- Dry the slide under exhaust hood for 30 minutes.
- Pre-heat an incubator and a thermocycler to 85°C.
- Mix 1 µl PNA probe in 100 µl hybridization buffer to a final concentration of 500 nM for each slide.
- Pre-warm the slide in a metal box lined with wet tissue paper in the pre-heated incubator for 5 minutes.
- Heat the diluted PNA probe in the thermocycler for 5 minutes.
- Remove the diluted PNA probe and put the tube immediately in the ice bath.
- Put the pre-warmed slide onto ice plate.
- Add 75 µl of diluted PNA probe to slide, cover immediately with a plastic strip.
- Incubate the cells in the metal box in pre-heated incubator for 10 minutes.
- Incubate the slides in room temperature for 2 hours.
- Remove the plastic strips.
- Incubate the slides in wash solution twice at 60°C for 10 minutes each.

- Incubate the slides in room temperature wash solution for 2 minutes.

Counter-staining with DAPI

- Add 50 µl DAPI solution to each slide and cover immediately with a plastic strip.
- Incubate the slides in room temperature for 10 minutes.
- Remove the plastic strip.
- Incubate the slides twice in 1X PBS for 2 minutes each.
- Shake the slides dry.
- Add 80 µl mounting medium and cover the slides with coverslips.

Imaging in confocal microscope

- Setup the confocal microscope laser for DAPI and Cy3 staining (pre-programmed in the Zen software).
- Put the slides on the microscope table and choose 63x objective.
- Set the Bits per Pixel to 16, Averaging at 2x and Dimension to 1024 x 1024.
- Adjust the z-stack setting to cover all the levels of the nuclei.
- Adjust the step size to 0.6 µm.
- Start the z-stack experiment.
- A minimum of 40 nuclei needs to be imaged per slide and per condition.
    - Do not change the settings during imaging of whole experiment.

Telomere length measurement

The measurements are using an in-house software from working group of Prof. Christian Bär and Dr. Shambhabi Chaterjee from Medizinische Hochschule Hannover (unpublished).