Institute of Pharmacology and
Toxicology

Section of Morphological and Behavioral Neuroscience

Protocol for non-radioactive in situ hybridization

S. Pfister, I. Knüsel, C. Köster, J.-M. Fritschy
Revised July 2012

Abbreviations
dH_2O, bi-distilled water
PBS, phosphate buffered saline
PFA, paraformaldehyde
RNA !, RNase-free
RT, room temperature

RNase-free conditions: - Zap bench with RNase Inhibitor spray
- all buffers prepared with DEPC-water
- stock buffers autoclaved
- glas and metal instruments heat sterilized (180°C in oven, 2h)
- plastics UV-sterilized
- work always with gloves and steril pipets

Day 1: Preparing tissue

- Anaesthetize mouse, decapitate and quickly dissect the tissue of interest
- Snap-freeze the tissue on powdered dry ice. If needed, embed it first in aluminum foil filled with Neg Frozen section Medium (Richard-Allan scientific, MI, USA)
- Storage at -80°C for up to 1 month
- Cut sections with a cryostat (15-20 µm) and mount on SUPERFROST PLUS gold glass slides (Menzel GmbH & Co, Braunschweig)
- Let them dry 1 h at RT and store at -80°C for 24-72 h

Note: if you work with sections for fixed tissue, mount them on the slides with a Pasteur pipet and let dry overnight; skip the fixation step below

Day 2: Preparing for hybridization

- Take the slides out of the freezer and air-dry for 30- 60 min at RT
- Alkaline fixation: Fix tissue sections at 4°C for 30 min in 4% PFA in 0.1 M $NaHCO_3$/0.1 M Na_2CO_3 pH :9.5 (8g PFA in 200 mL Solution, ¾ $NaHCO_3$, ¼ Na_2CO_3)
- Wash 2 times in PBS for 5 min
- Optional: DEPC treatment: 50% EtOH + 0.5% DEPC (50 mL EtOH + 50 mL dH_2O + 500 uL DEPC; prepare fresh under the hood); Incubation 30 min at RT
- wash 2 times in PBS for 5 min
- Incubate in 0.02 M HCl for 10min (400 µL 10 N HCl + 200 mL DECP-H_2O)
- Wash in PBS for 5 min
- Optional: Incubate in 0.01% Triton-X100 dissolved in PBS for 3 min (20 µL Trition-X-100 in 200 mL PBS)
- Wash in PBS for 5 min
- Treat sections with Proteinase K (about 400 µL/slide) for 10 min at RT in humid chamber

Proteinase K (Roche) Stock solution: 20 mg/mL
- Long probes (up to 2000 bp): 5 µg/mL Proteinase K in 50 mM Tris and 5 mM EDTA pH 7.4
- Short probes (up to 800 bp): 0.5 µg/mL Proteinase K in 50 mM Tris and 5 mM EDTA pH 7.4 (dilute 5 ug/mL Proteinase K 1:10).

- Wash in PBS for 5 min
- Optional: Acetylation (can be done in addition to or instead of proteinase K treatment; test for best results)

  Place a glass slide-container on a stirrer with a magnet. Fill with 0.1 M Triethanolamine and 20 mM HCl, mix well and stir constantly.

  Insert the slides and add 750µL/100 mL acetic anhydride, drop after drop, over the slides, still stirring.
  Incubate for 10 min. (stirr all over this time)

  Wash in 2x SSC

- Prehybridization in hybridization buffer at 55°C for around 1h:
  About 200 µL/slide, placed in humidified chamber

  For 20 mL:
  - 10 mL formamide
  - 5 mL SSC10x
  - 500 µL total yeast RNA (2.5 mg)
  - 1 mL Herring sperm DNA (5 mg)
  - 2 mL Denhardt's solution

  Humidified chamber: 5x SSC+ 50% Formamide

- Dilute DIG-labeled RNA in hybridization buffer (200-800 ng/mL)

  For 20 mL:
  - 10 mL formamide
  - 5 mL 10x SSC
  - 500 µL total yeast RNA (2.5 mg)
  - 1 mL Herring sperm DNA (5 mg)
  - 2 mL Denhardt's solution
  - 2 g Dextran Sulfate (1%)

- mix RNA hybridization buffer at 75°C for 5 min
- Put it immediately on ice
- Pipet around 200 - 300 µL RNA Hybridization buffer per slide

  Cover them with HybriSlips
- Incubate at hybridization temperature overnight

Days 3-4 Detection post-hybridization

- Warm up 5x SSC, 1x SSC and 0.1x SSC to hybridization temperature
- Prepare 1% blocking solution:

  1 g blocking reagent in 100 mL detection buffer

  dissolve in microwave oven by successive steps of 20 sec
- Never let boil! Mix and cool down
- Wash slides in 5x SSC for 5 min RT
- Wash slides in 5x SSC for 10 min at hybridization temperature
- Wash slides in 1x SSC for 10 min at hybridization temperature
- Wash slides in 0.1x SSC for 20 min at hybridization temperature
- Wash slides in 0.1x SSC for 5 min RT
- Wash slides 2 times in 1x detection buffer for 5 min RT
- Equilibrate slides in 1% blocking buffer for 1 hour RT (keep some blocking buffer to prepare anti-DIG solution!!!)

Follow distinct protocols for detection with alkaline phosphatase or with fluorescent probes (CatFISH)

A) Detection with alkaline phosphatase

- Dilute anti-DIG antibody 1:4000 in blocking buffer (200 µL/slide)
- Encircle the sections with liquid blocker pen (SCI, science service, Munich, Germany). Put antibody solution on the sections (200 µL/slide)
- Incubation at 4°C overnight in humid chamber

On the following day:

- Wash slides 2 times in 1x detection buffer for 15 min RT
- Prepare developing solution (per 1 mL):
  - 100 µL AP10x
  - 50 µL $MgCl_2$
  - 4.5 µL NBT
  - 3.5 µL BCIP
  - 10 µL Levamisol
  - 832 µL dH_2O

  Filter with 0.45 µm-filter and keep in the dark

- Equilibrate with 1x AP/$MgCl_2$ for 5 min (1:20 $MgCl_2$)
- Pipet around 300 µL developing solution on each slide and incubate in humidified chamber at RT in the dark until proper color is reached (reaction time: 3-24 h)
- Wash 3 times in PBS at least for 15 min
- Optional: Stop reaction in 0.2 M EDTA (pH: 8.0)

B) CatFISH detection (Roche diagnostics)

- Dilute primary antibodies (e.g., anti-DIG POD 1:5000 / anti-NeuN 1:1000) in blocking buffer + 5% NGS (200 µL/slide)
- Encircle the sections with liquid blocker pen (SCI, science service, Munich, Germany). Put antibody solution on the sections (200 µL/slide)
- Incubate at 4°C overnight in humid chamber

On the following day:

- Wash 2 times in 1x TNT buffer for 15 min RT
- Add TSA Working Solution: TSA Cy3 stock solution 1:50 in Amplification Diluent
- Incubate 3-10 min at RT
  Note: most likely sections need to be encircled again with blocker pen
- Wash 3 x 5 min in TNT
- Wash 1 x 5 min in Tris-Triton (0.05% Triton X-100) pH 7.4
- Incubate 1h with 2nd antibody (e.g., goat anti-mouse Alexa 488, 1:1000 in Tris-Triton pH 7.4 + 4% NGS + 0.2% Triton)
  Note: most likely sections need to be encircled again with blocker pen
- Wash 3 x 10 min in PBS
- Dip slides in dH_2O
- Coverslip with 50 µL ProLong Gold Antifade Reagent (with DAPI)
- Store in the dark at 4°C

SOLUTIONS FOR IN SITU HYBRIDIZATION

DEPC-H_2O

Compounds

- Bidistilled water, dH_2O
- DEPC: Sigma D-5758

1ml DEPC (diethyl pyrocarbonate) in 1L UHP water

Stirr overnight under the hood. Autoclave 45 min.

PHOSPHATE-BUFFERED SALINE

Compounds

- Bidistilled water, dH_2O
- Sodium chloride, NaCl, Fluka, puriss, Mr = 58.44 g/mol
- Potassium chloride, KCl, Sigma, Mr = 74.551 g/mol
- Sodium hydrogen phosphate monohydrate, Na2HPO4. H2O, Sigma, Mr = 118.97g/mol
- Potassium dihydrogen phosphate, KH_2PO_4, Merck, Mr = 136.08 g/mol

20x PBS-buffer (stock)

NaCl	320 g
KCl	8 g
Na_2HPO_4. H2O	57.7 g
KH_2PO_4	8 g
dH_2O	2000 mL

Adjust the pH to 7.4 with HCl

Add 2 mL DEPC and stir overnight. Autoclave for 45 min.

SALINE SODIUM CITRATE BUFFER (SSC)

Compounds

- Bidistilled water, dH_2O
- Sodium chloride, NaCl, Fluka, puriss, Mr = 58.44 g/mol
- Tri-sodium citrate dehydrate, $C_6H_5Na_3O_7 \cdot 2H_2O$, Fluka, puriss, Mr = 294.10 g/mol
- Diethyl dicarbonate, DEPC, $O(COOC_2H_5)_2$, Sigma, Mr = 162.14 g/mol

20x SSC (stock)

0.3 M $C_6H_5Na_3O_7 \cdot 2H_2O$	176.5 g
3 M NaCl	350 g
dH_2O	2000 mL

Adjust the pH to 7.4 with HCl

Add 2 mL DEPC and stir overnight. Autoclave for 45 min.

PRE- AND HYBRIDIZATION SOLUTION

Compounds

- Bidistilled water, dH_2O
- Denhardt's solution 50x, Invitrogen, Ultrapure
- Formamide, $HCONH_2$, Fluka, puriss, for molecular biology, Mr = 45.04 g/mol
- Herring sperm DNA, Invitrogen, Ultrapure
- RNA from yeast, Roche
- Dextran sulfate sodium salt from Leuconostoc spp, Sigma

Prehybridization solution (20 mL)

	Formamide	10 mL
	10x SSC	5 mL
	Denhardt's solution	2 mL
2.5 mg	Herring sperm DNA	1 mL
5 mg	RNA from yeast	500 µL
	dH_2O	1500 µL

Hybridization solution (20 mL)

	Formamide	10 mL
	10x SSC	5 mL
	Denhardt's	2 mL
2.5 mg	Herring sperm	1 mL
5 mg	RNA from yeast	500 µL
1 %	Dextran sulfate	2 g
	dH_2O	1500 µL

10X DETECTION WASH BUFFER

Compounds

- Bidistilled water, dH_2O
- Sodium chloride, NaCl, Fluka, puriss, Mr = 58.44 g/mol
- Tris-(hydroxymethyl)-aminomethane, $C_4H_{11}NO_3$, Fluka, puriss, Mr = 121.14 g/mol

10x Detection wash buffer (stock)

1 M	$C_4H_{11}NO_3$	121.14 g
1.5 M	NaCl	87.6 g
dH_2O		800 mL

Adjust the volume to 1 L with dH_2O and the pH to 7.5 with HCl. Autoclave for 45 min.

10X ALKALINE PHOSPHATASE BUFFER (AP)

Compounds

- Bidistilled water, dH_2O

- Sodium chloride, NaCl, Fluka, puriss, Mr = 58.44 g/mol
- Tris-(hydroxymethyl)-aminomethane, $C_4H_{11}NO_3$, Fluka, puriss, Mr = 121.14 g/mol

10x Alkaline phosphatase buffer (AP) (stock)

1M	$C_4H_{11}NO_3$	121.14 g
1M	NaCl	58.44 g
dH2O		800 mL

Adjust the volume to 1 L with dH_2O and the pH to 9.5 with HCl. Autoclave for 45 min.

1% BLOCKING REAGENT SOLUTION

Compounds

- Blocking reagent, Roche
- 1X Detection buffer

Solve in microwave oven by successive steps from 20 sec. Mix in between and never let boil. Cool down to RT before use.

1M $MgCl_2$

203.3 g MgCl2.6H2O in 1000mL dH2O. Autoclave for 45 min.

Levamisole

Compounds

- Levamisole hydrochloride salt, Sigma L9756

Dissolve 24 mg/mL in AP buffer. Store 200 µL aliquots at -20°C.

Developing solution

Compounds

- 10X AP buffer
- 1 M $MgCl_2$
- nitroblue tetrazolium salt, NBT, 75 mg/mL, Roche
- 5-bromo-4-chloro-3-indolyl phosphate, BCIP, 50 mg/mL, Roche
- Levamisole hydrochloride salt, 24 mg/mL in AP buffer, Sigma

Developing solution (1 mL)

10x AP	100 µL
MgCl2	50 µL
NBT	4.5 µL
BCIP	3.5 µL
Levamisole	10 µL
dH_2O	832 µL