Protocol for Fluorescence In Situ Hybridization coupled with Tyramide Signal Amplification step (FISH-TSA)

Dr. Vladimir Krylov, Department of Cell Biology, Faculty of Science, Charles University in Prague, Czech Republic

This protocol is intended for the visualization of single copy genes on Xenopus chromosomes

1. Preparation of slides

- prepare cells as usual (trypsinization, hypotonization, prefixation and fixation three times by methanol: acetic acid 3:1)

- drop the cells onto clean microscopic slides from 10 cm height

- put the slide on plate warmed up to 37 °C

- let the suspension dry and observe the surface

- when the surface seems to be like sand immerse the slide in 50% acetic acid for 2 seconds

- wipe the bottom side of slide and let dry out on plate (37 °C)

- after drying put slides for 5 min to the pepsin solution (50 μg/ml 0.01 M HCl, 37 °C) **BE CAREFULL !!!!** Pepsin lost its activity if pH is higher then 7.0. Prepare pepsin stock solution (1 mg/ml in 3 mM acetate buffer, pH = 4.6). Then dilute this stock 20x by warm (37 °C) 0.01 HCl and let warm up for 5 min before incubation of slides

- take out slides from the pepsin and immerse them in PBS (phosphate buffer saline, pH = 7.3). This step inactivates the pepsin activity

- wash slides by PBS with agitation for 5 min (in 20 cm glass Petri dish filled with approx. 100-150 ml of PBS)

- immerse slides in 2% paraformaldehyde solution (2% paraformaldehyde in PBS) for 30 min (RT)

- take out slides and wash them three times with PBS with agitation in Petri dish

- immerse slides in 1% hydrogen peroxide (1% H_2O_2 in PBS) for 30 min (RT). This step is important for the inactivation of endogenous peroxidase activity

- take out slides and wash them three times by PBS with agitation in Petri dish

- rinse slides by distillate water and dehydrate them by increasing concentration of methanol (70, 90 and 100%), 3 min for each concentration with agitation in Coplin jar

- let slides dry out

- in this time you can put slides to freezer (-20 °C) and store them at least 2 month and may be longer

2. Preparation of the probe

We always use cDNA probe of approx. 1000 bp in length depending on gene of interest. Any repetition could make a strong background using TSA

- prepare at least 2 µg of cDNA amplificate from reverse transcription reaction by PCR and specific primers

- purify the amplificate through agarose gel and column of Gel Extraction Kit (QIAGEN)

- label this cDNA by Digoxigenin-11-dUTP alkali stable (Roche, cat.no. 11 558 706 910) and DecaLabel DNA Labeling Kit (Fermentas, cat.no. K0622) – labeling by random primers. We always label 1µg of cDNA for 20 hours. After this time we purify the probe with column of Gel Extraction Kit (QIAGEN) without using an agarose. Just mix probe with binding buffer. Elution is done with 50 µl of sterile water. We always use 2 µl of probe in 50 µl reaction per one slide

3. FISH-TSA – day 1

- take out slides from the freezer and let dry them out

- prepare the hybridization mixture (volume per one slide):

25 μl deionized formamide (Sigma - F9037)
2 μl of cDNA probe
5 μl 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM sodium citrate, pH = 7.0)
18 μl sterile deionized water

50 μl TOTAL

- apply 50 μl of hybridization mixture on slide and cover by cover glass (24 x 50 mm)
- denature slides at 70 °C for 5 min in thermocycler with plate adapter

- transfer slides immediately into wet chamber warmed up to 37 °C and let hybridize in incubator (37°C) at least 12-16 hours (overnight)

4. FISH-TSA – day 2

- take slides out from the wet chamber and wash them (move up and down) three times in washing solution (50% formamide in 2 x SSC, pH = 7.0) for 5 min each (42 °C). We always use three coplin jars immersed in water bath (42 °C) and filled with 45 ml of washing solution for 4 slides

- wash slides three times by 2 x SSC for 5 min with agitation at RT in Petri dish

- wash slides by 1 x TNT (0.1 M Tris/HCl, 0.15 M NaCl, 0.05% Tween 20, pH = 7.5) for 5 min with agitation (RT) in Petri dish

- wipe the bottom side of slides and put them in wet chamber

- apply 1 ml of blocking buffer (TNB – 0.1 M Tris/HCl, 0.15 M NaCl, 0.5% Blocking reagent) per one slide and incubate for 30 min at RT

- pour off blocking buffer and apply 1 ml antidigoxigenin-POD antibody (Roche, cat.no. 11 207 733 910) diluted 1:1000 with TNB buffer per one slide. Incubate 1 hour in wet chamber (RT)

- pour antibody and rinse slides three times by TNT

- wash slides three times by TNT for 5 min with agitation in Petri dish

- take slides out and wipe the bottom

IMPORTANT: don´t let slides dry out at this time !

- apply 100 µl of tyramide solution per slide and cover by cover glass (24 x 50 mm). Incubate for 10 min in dark (RT)

We use TSA kit from Perkin Elmer company (cat. no. NEL 741 – fluorescein or NEL 742 – Rhodamine). The company is very important. Other kits are not so good as from Perkin Elmer is.

- rinse slides three times by TNT (let the cover glass slip out)

- wash slides three times by TNT for 5 min with agitation in dark in Petri dish

- rinse slides with distilled water and wash them 3 min by distilled water with agitation in Petri dish

- take out slides and let dry them out in dark

- mount slides for fluorescence microscopy (Mowiol/DAPI or similar)

- you can store slides more then one week in 4 °C in dark without loss of activity of TSA signals.

GOOD LUCK !!!!!!!

Literature:

Khokha M.K., Krylov V., Reilly M.J., Gall J.G., Bhattacharya D., Cheung C.Y., Kaufman S., Lam D.K., Macha J., Ngo C., Prakash N., Schmidt P., Tlapakova T., Trivedi T., Tumova L., Abu-Daya A., Geach T., Vendrell E., Ironfield H., Sinzelle L., Sater A.K., Wells D.E., Harland R.M., Zimmerman L.B. Rapid gynogenetic mapping of Xenopus tropicalis mutations to chromosomes. **Dev Dyn**. 2009; 238(6):1398-1406.

Krylov V., Tlapakova T., Macha J. Localization of Mdh2 single copy gene on *Xenopus tropicalis* chromosomes by FISH-TSA technique. **Cytogenet. Genome Res.** 2007; 116:110-112.

Tlapakova T., Krylov V., Macha J. Localization, structure and polymorphism of two paralogous *Xenopus laevis* mitochondrial malate dehydrogenase genes. **Chromosome res.** 2005; 13(7):699-706.

Krylov V., Macha J., Tlapakova T., Takac M., Jonak J. The c-SRC1 gene visualized by in situ hybridization on *Xenopus laevis* chromosomes. **Cytogenet. Genome Res.** 2003;103(1-2):169-172.