If you use this protocol, please reference: Subhashree Ganesan, Julie Karr, and David E. Featherstone. (2011) Drosophila glutamate receptor mRNA expression and mRNP particles. RNA Biology 8(5):1-11

Fluorescent In Situ Hybridization with DIG-RNA probes on dissected larval preps

RNA probe preparation

1. Invitro transcription (from Roche kit)
   Set up following reaction on ice.

Linearized template DNA (1ug)	= 13ul*
10X NTP mix	= 2ul
10X Transcription buffer	= 2ul
RNase inhibitor	= 1ul
RNA polymerase	=2ul

   *(Make up volume to 12µl with nuclease free non-DEPC water because residual DEPC can inhibit transcription)

   Mix by pipetting.
   Centrifuge briefly to collect at the bottom.
   Incubate at 37C for 2 hours.
   (Longer incubations do not increase yield-Roche)

2. Add 2ul DNaseI. Incubate for 15 minutes at 37°C.
3. Stop the reaction by adding 2ul of 0.2M EDTA (pH8.0) OR directly proceed to Alkaline hyrolysis.
4. Precipititate RNA by LiCl precipitation (below) and resuspend in 50µl nuclease free water.
4. Run agarose gel to check size

Partial alkaline hydrolysis of RNA probe

1. If RNA is from step 2 above directly, add 3µl H_2O to make up volume to 25µl.
2. To 25µl of probe, add 25µl of 2X carbonate buffer (recipe at the end).
3. Incubate at 60°C for desired length of time
   (Can divide into two halves and incubate one for 5 mins and other for 15 minutes. 15 mins works well for RNA that are ~3kb. It gives an average size of 100-150bp after hydrolysis).
4. Add 50µl Stop Solution.
5. Precipitate RNA by Lithium Chloride precipitation.
6. Check size by Agarose Gel Electrophoresis

Lithium Chloride precipitation of RNA (Roche kit)

1. To RNA in a volume of 100µl, add, 10µl 4M LiCl, 250µl Ice cold ethanol. Mix
2. At least 2 hours at -20°C (Safe to leave overnight).
3. Centrifuge at 4°C, 15 minutes, 13000g.
4. Decant and wash with 50µl ice cold 70% ethanol.
5. Centrifuge at 4°C, 5 minutes, 13000g.
6. Resuspend in freshly prepared resuspension buffer.
7. Store at -80°C for long term storage, at -20°C otherwise.

RNA Gel Electrophoresis

1. Rinse casting tray, comb, gel tank with RNase ZAP and rinse with DEPC water.
2. Cast 2% agarose gel in RNAse free 1X MOPS-EDTA-Sodium Acetate buffer. Do not add EtBr, loading buffer has EtBr.
(10X buffer from Bioline, dilute treat with DEPC O/N, autoclave. OK if yellow color appears after autoclaving.)
3. Add equal volume of 2X RNA loading buffer with Ethidium Bromide (bioline) to samples and to 4µl RNA ladder (if needed).
4. Denature at 65°C for 10 mins, back on ice for a minute.
5. Load and run at 60V-100V.
6. Based on what Stephan Sigrist's protocol says:, if you barely see anything use 5µl, if there is a moderate brightness smear 3µl, and if very bright smear, 2 µl probe per 500µl hybridrization buffer. Smears are better than bands.

Hybridization protocol

(Based on protocol from Stephan Sigrist, protocol on BDGP expression analysis project and; Braissant and Wahli, Biochemica,1998)

DAY1

1. Dissect wandering third instar larvae in Roger's ringer, on sylgaurd plates.
2. Fix for 10 minutes with fresh 4% Paraformaldehyde(PFA)/PBS (freshly prepared same morning) on the sylgaurd plate.*
3. Fix for 50 minutes in 4%PFA/PBS in tube in ice (Unpin and transfer larvae to tubes filled with the fixative). *
4. Rinse 3X in P.Tx (0.05% TritonX-100/PBS)*
5. Wash 1 hour in PTx with shaking at RT.*
6. Rinse in 500µl freshly prepared prehybridization buffer (-DS buffer).
7. Prehybridize at RT, shaker for around 2 hours or more.
8. Prepare hybridization buffer (+DS Hybridization buffer).
9. Denature probe at 80°C for 5 minutes, and directly add to the +DS hybridization buffer.
10. Incubate in probe solution at 55°C in a shaking incubator, for around 40 hours (I have even left it all weekend). Longer incubation gives better signal.

DAY3

11. Rinse 2X with freshly prepared wash buffer.
12. Wash 8X (30 minutes each) in pre-warmed wash buffer in the 55°C shaker.
13. Wash overnight in pre-warmed wash buffer at 55°C, shaker.

DAY4

14. Rinse 2X in PTw at RT.
15. Wash 30 minutes in PTw at RT.
16. Incubate O/N in 1:1500 αDIG antibody (and any other primary antibody for co-immunostaining) in 0.1%B.S.A/P.Tw at 4°C, shaker.

DAY5

17. Rinse 2X in PTw at RT.
18. Wash 8X 10 mins each in PBTw at RT, shaker.
19. Incubate 1.5-2 hours in secondary antibody in 0.1%B.S.A/P.Tw at RT, rocker.
20. Rinse 2X in PTw at RT.
21. Wash 8X (10 minutes each) in PTw at RT.
22. Mount with Vectashield and view.

*For dissected 1st instar larvae, transfer to cell culture dish after dissection and add cold 4% PFA/PBS and fix for an hour. Rinse 6X with PBTw and keep in PBTw for 1 hour at RT and then prehybridize and then same protocol as above.

Reagents and Solutions for hybridization protocol

2X Carbonate Buffer

127.2 mg sodium carbonate
67.2mg sodium bicarbonate
Add DEPC water to 10ml
pH to 10.2 with NaOH
Store in 1ml aliquots at -20C.

Stop solution

164.1mg sodium acetate
Add water to 10 ml
pH to 6.0 with acetic acid
Store in 1ml aliquots at -20C.

Resuspension Buffer(1ml)

500µl TE (10mM Tris HCl,500mM EDTA, pH 7.5, in DEPC water)
500µl Deionized formamide, 5µl Tween-20

4% Paraformaldehyde fixative solution (as in Maniatis 18.87)

Dissolve 4g of paraformaldehyde(sigma) in 50 ml of H_2O.
Add 1ml of 1M NaOH solution.
Stir the mixture gently on a heating block (~65°C) until the paraformaldehyde is dissolved.
Add 10 ml of 10X PBS (DEPC treated) and allow the mixture to cool to RT.
Adjust pH to 7.4 using 1MHCl (~1ml is needed).
Adjust volume to 100ml with DEPC H_2O.

P.B.Tx/DEPC (Phosphate Buffered Saline with 0.05% TritonX-100)

Dissolve 25ml 10X PBS in 225ml H20 to make 250ml PBS solution
Add 250µl DEPC and incubate at 37°C overnight.
Autoclave for 20-30 mins to destroy DEPC.
After it cools down add 125µl TritonX-100.

P.B.Tw/DEPC (Phosphate Buffered Saline with 0.1% Tween-20)

Make 250 ml PBS solution like above and treat with DEPC.
After it cools down from autoclaving add, 250µl Tween-20.

Prehybridization buffer or -DS hybridization buffer (without dextran sulfate)

Must be freshly prepared each time.
To make 2 ml –DS hybridization buffer:

1ml- Deionized formamide (Sigma)
400µl-20XSaline Sodium Citrate buffer (Sigma)
40µl-50X Denhardt's Solution (Invitrogen)
50µl-tRNA (yeast tRNA from Invitrogen, 10mg/ml stock solution)
50µl sheared salmon sperm DNA (10mg/ml stock Ambion, before adding, heat at 100°C for 10 mins and chill on ice)
2µl Heparin (50mg/ml, made stock and store at 4°C)
10µl- Tween-20
448µl-DEPC water

+DS hybridization buffer (with Dextran Sulfate)

Must be freshly prepared each time.
To make 1 ml +DS hybridization buffer:

500µl- Deionized formamide (Sigma)

200µl-20X SSC (Sigma)
20µl-50X Denhardt's Solution (Invitrogen)
25µl-tRNA (yeast tRNA from Invitrogen, 10mg/ml stock solution)
25µl sheared salmon sperm DNA (10mg/ml stock Ambion, before adding, heat at 100°C for 10 mins and chill on ice)
1µl Heparin (50mg/ml)
5µl- Tween
124µl-DEPC water
100µl-50% Dextran sulfate (Amresco)

Wash buffer

10 ml Wash buffer

Deionized Formamide- 5ml
20XSSC -1ml
DEPC WATER - 4ml
Tween 20 -50µl
0.2M EDTA, pH8

In about 85-90ml of Nano water dissolve 7.44 grams of EDTA-disodium salt (FW 372.2)
With NaOH adjust the pH to 8.0.
Make up the volume to 100ml.
Add 100ul of DEPC. (0.1%) Leave overnight at 37C.
Autoclave for atleast 15 minutes on liquid cycle.

4M Lithium Chloride

Dissolve 16.96 gms of Lithium Chloride in 90ml of water and make up volume to 100 ml.
Treat with 100ul DEPC at 37C overnight and autoclave.

10mg/ml yeast tRNA solution in nuclease free water

Invitrogen yeast tRNA comes in 25mg per bottle. Add 2.5ml nuclease free water and dissolve. Aliquot 500µl per tube and store at -20°C. Minimize freeze thaw.