Kathy Su

Host Lab: Bowsher Lab

Date of Exchange: Oct 2013 – Nov 2013

IN SITU HYBRIDIZATION IN THEMIRA BILOBA PUPA

Sampling

Pick light brown prepupa from dung plates and place in a petri dish.
At this stage there is a visible gas bubble within the abdomen.
This stage can last for about 12 hours depending on sepsid species.

Wash the pupae immediately by adding distilled water into the petri dish and placing it on a standard analog shaker.

NB: It is necessary to wash the pupae NOW, as washing them later in development only allows the dung to harden, after which it is almost impossible to effectively remove the dung from the pupae.

Stick double-sided tape onto a glass slide and arrange the washed pupae onto the tape.

Place the pupae in an incubator set at 25°C.

Staging the Pupae (at 25°C)

48 hours	Young pupae imaginal head sac is everted and oral armature of larva is expelled
72 hours	Eye cup is yellow at the perimeter
90-96 hours	Eyes are red but no pigmentation has formed

Dissection and Fixation

Slice pupae quickly along the Dorsal/Ventral axis with a sharp scalpel.
Pick up each slice from the pupal case gently and place them in a petri dish with distilled water
Using a pipette, very gently flush water over the pupae to remove excess fat bodies.
Fix pupae in 4% PFA-PBT for 15 min at RT on a shaker.
Wash 3 x in 100% MeOH.
Store tissues in 100% MeOH at -20°C.

Rehydration

Rehydrate at RT on a shaker

70 % MeOH : 30% PBT 5 min
50 % MeOH : 50 % PBT 5 min
30 % MeOH : 70 % PBT 5 min

Wash in PBT 3 x 5 min

Do not carry out a proteinase K treatment for pupae.

Hybridization

Prepare Hyb buffer with block (HBB)

Equilibrate in 500µl of 1:1 PBT:HBB for 10 min
Do not nutate to let the tissues settle

Remove 1:1 and replace with HBB, incubate for 10 min

Heat treat in hot block at 75°C for 40 min in HBB

Prehybridize for 2-3 hours at 67°C in a water bath
Ensure that tissues are fully submerged in the water

Dilute probes in HBB to a final concentration of 0.1-0.3 µg/ml

Denature the probes by incubating at 80°C for 5 min, then chill on ice

Remove HBB from tissues and replace with denatured probe

Hybridize >12 hours at 67°C in a water bath

Antibody staining

Prewarm Hybridization buffer without block (HB) in the water bath at 67°C.

Remove the probe from the tissues and replace with 1ml of prewarmed HB.

Wait for the tissues to settle.

Remove the HB again and replace with 1ml of prewarmed HB.

Wash for 2-3 hours at 65°C, replacing the HB every hour.

Wash for 30 min at 65°C in 500µl of 1:1 HB:PBT.

Wash in PBT for 3 x 5 min at RT.
Place tubes sideways on a shaker.

Block the tissues by washing 3 x 10 min in 2% BSA Blocking Solution.

While tissues are blocking, dilute anti-DIG-AP antibody 1:2000 in the Blocking Solution.

Incubate tissues overnight at 4°C.

Developing

Remove the antibody solution.

Rinse 3x in PBT.

Wash at least 6 x 10 min in PBT.
Place the tubes on a slow shaker during the washes.
Extended washing periods will decrease background.
Washing can be done over the course of a day or even overnight.

Wash 2 x 5 min in Alkaline Phosphatase Developing Solution (DS).
Remember to add Tween to 0.1% to the developing solution stock.
500 μl of 10 % Tween in 50 ml stock.

Transfer tissues to a cell culture plate in 1ml of DS.

Develop with 4.5 μl of NBT (75 mg/ml in 70% dimethylformamide (DMF)) and 3.5 μl X-Phosphate (50 mg/ml in DMF) per 1 ml of DS.

Develop in the dark.

Stop the reaction by washing well in PBT.

Wash again in 100% EtOH and store tissues in -20°C until ready to mount.

REAGENTS

All reagents must be RNAse-free.

PFA-PBT 4%

10ml	16% PFA (Electron Microscopy Science)
400µl	10% Triton-X
4ml	10X PBS
25.6ml	H_2O

PBT

100ml	10X PBS
1ml	Triton-X
900ml	H_2O

Hybridization Buffer

50% Formamide
250 ml of 100 % Formamide stored at 4°C
5X Denharts
50 ml of 50X Denharts stored at -20°C
5X SSC
125 ml 20X SSC stored at RT
0.1% Tween
5 ml 10% Tween stored at RT

DEPC H_2O
70 ml

Hybridization Buffer with Block

Mix in 50 ml falcon tube and store at -20°C

Hybridization Buffer	50 ml
tRNA (50 mg/ml stock for final concentration of 250 µg/ml	200 µl
Heparin 100 mg/ml stock for final concentration of 100 µg/ml	50 µl
Salmon sperm 10 mg/ml stock for final concentration of 200 µg/ml	100 µl

Blocking Solution (2% BSA in PBT)

1g	BSA
50ml	PBT

ANTIBODY STAINING IN T. BILOBA LARVAE HISTOBLAST

Sampling

Pick gut purged larvae and place on a moist filter paper to remove dung.
Take larva and place in -20°C for 2 min (not more) to immobilise it straight.

Dissection

Dissect larva on ice.
Snip off spiracles and mouth hooks with micro scissors.
Arrange larva dorsal side up, cut from posterior to anterior in between the trachea.
Larva opens up flat.
Remove gut and trachea tissues.

Fixtation & Storage

Fix the larva by placing it into 4% PFA- to fix for 15 min at RT.
Wash 3 times with 100% methanol.
Store in 100% methanol at -20°C.

Antibody Staining Protocol

Rehydrate at RT on a shaker

70 % MeOH : 29.5 % PBT : 0.5 % H_2O_2	30 min
50 % MeOH : 50 % PBT	5 min
30 % MeOH : 70 % PBT	5 min

Rinse in PBT 3 x 5 min
Wash in PBT 1 x 30 min

Block for 1 hour at RT in PBT+N (4.75ml PBT, 0.25ml goat serum)
NB: Keep PBT-N at 4°C for not more than 1 week.

Incubate overnight with primary antibody at 4°C or RT for 2-3 hours.

Rinse in PBT 3x
Wash in PBT 6 x 20min at 4°C

Incubate with secondary antibody overnight at 4°C
596µl of PBT+N and 4µl of HRP conjugated secondary antibody

Rinse in PBT 3x
Wash in PBT 6 x 20min at 4°C

Developing in DAB stain

Place larvae into cell culture plates

Prepare developing stain:
Add 8μl of NiCl to DAB aliquot (1ml solution of DAB 300mg/ml)

Add 300μl of developing stain to larvae and incubate 10 min in the dark

Add 1-2μl of H_2O_2 and watch for colour to develop
Usually takes about 5-10 min

Wash 4-5x in PBT to stop the reaction

Mount and check for expression in histoblast cells

REAGENTS

PFA-PBT 4%

10ml	16% PFA (Electron Microscopy Science)
400µl	10% Triton-X
4ml	10X PBS
25.6ml	water

PBT

100ml	10X PBS
1ml	Triton-X
900ml	water