In situ hybridization for Gryllus embryos (10/03/2008)

Fixed embryos are stored in 100% MeOH at -20˚C, all steps are done in 1.5 ml tubes
Prepare PT (2l), MeOH/PT, Hyb solution 1

Day 1 (5h)

Pre-treatment

1) **Rehydration**
   - Wash 1x10', 50 % MeOH / PT ○
   - Wash 2x5', PT ○ ○

2) **Permeabilization**
   - Wash 1x 5' in PT-ProK (2µg/ml) ○
   - Wash 2x 5' in PT ○ ○

3) **Post-Fixation**
   - 1x20' n PT-FA ○
   - Wash 1x 1', 2x 5' in PT ○ ○ ○

Hybridisation

4) **Pre-hybridization**
   - 1x 5' PT/50% Hyb solution A ○
   - 1x 10', 2' 60' (min. 30', can be 2-3h) in 100% hyb solution A at 60˚C ○ ○ ○

   Comments:
   - mix embryos with a finger tap a couple of times
   - embryos can be stored in hyb solution at -20C

5) **Hybridization**
   - Aliquot embryos to about 50µl per eppendorf
   - Dilute probe in 100ul hyb solution (mostly 1ul of probe stock is sufficient)
   - Boil the probe for 5' to denature; immediately chill on ice
   - Add the probe to the embryos and keep them at 60˚C over night (note time ○)
   - Mix embryos with a finger tap a couple of times.

Prepare for day 2: hyb solution B, PTB

Day 2

6) **Washes**
   - 1x 60 min prewarmed hyb solution B at 60˚C ○
   - 3x 30min Wash 1 (2x SSC) at 60˚C ○ ○ ○
   - 3x 30min Wash 2 (0.2x SSC) at 60˚C ○ ○ ○
   - 3x 10min PT at RT ○ ○ ○
   - 3x 1h PT at RT ○ ○ ○

7) **Blocking step**
   - Wash 1x 10', 1x 60' (or longer) in PTB ○ ○

8) **Primary antibody**
   - Incubate PTB/mouse α–DIG-AP (1:1000) O/N at 4˚C ○

Day 3

9) Equilibration & AP staining reaction

- 2x 1 min PT ○ ○
- 6x 30 min PT ○ ○ ○ ○ ○
- prepare NTMT (9ml/probe) and AP buffer
- 1x AP-buffer for 15' ○
- AP-buffer and NBT/BCIP at RT or 4˚C in the dark ○

10) Stopping AP reaction

- 5x 5min in PT ○ ○ ○ ○ ○

11) Decolorization (optional)

- 50% EtOH/PT at RT: variable time ○
- 100% EtOH ○
- 50% EtOH/PT at RT: variable time ○
- 1x 5' in PT ○

Comments: keep examining the embryos using a stereoscope to avoid over-decolorization. EtOH reacts with AP reaction solution resulting in precipitation.

12) Clearization

- 25% Glycerol/PT until embryos sink ○
- 50% Glycerol ○

Comments: use 50% if embryos should be mounted individually (easier to pipette..)

Appendix: Solutions

- PBS(pH7.4)
  a. 10x PBS 100ml
  b. DEPC H_2O 900ml

- PT(pH7.4) (PBS + 0.1% Triton X-100)
  a. PBS 1L
  b. Triton X-100 1mL
  - Keep stirring the prepared solution O/N, because Triton X-100 is viscous.

- PT-FA (pH7.4) (PBS + 0.1% Triton X-100 + 4% Formaldehyde)
  a. PBT 18 ml
  b. 40% Formaldehyde 2 mL
  - Keep stirring the prepared solution O/N, because Triton X-100 is viscous.

- PT-ProK (PT+ 2µg/ml Proteinase K)
  a. ProK stock solution (20mg / mL) 1µL
  b. PT 10mL
  - Prepare for each use and keep on ice. Use stock from Invitrogen

- PTB (PT+ 1.5% Roche Blocking reagent)
  a. Western Blocking Reagent (10%) 1.5 ml
  b. 10x PBS 1 ml
  c. 20% Triton X-100 50 µl
  d. DEPC H2O 7.45 ml

  - Dissolve and store aliquots at -20°C.

- Hybridization Mix A
  De-ionize Formamide:
  Add 4g mixed red resin to 40 ml Formamide in 50 ml Falcon tube and shake for 1h, pour formamide in new tube in such a way that red resin stays in old tube

	for 10ml		**for 20ml**		**for 50ml**	
Roche Blocking solution (final 2%)	2	ml	4	ml	10	ml
Formamide (final 50%)	5	ml	10	ml	25	ml
20xSSC (pH 7.0) (final 5x)	2.5	ml	5	ml	12.5	ml
10% TritonX-100 (final 0.1%)	0.1	ml	0.2	ml	0.5	ml
10% CHAPS (final 0.1%)	0.1	ml	0.2	ml	0.5	ml
0.5M EDTA (final 5mM)	0.1	ml	0.2	ml	0.5	ml
Heparin (50 mg/ml) (final 50 µg/ml)	10	ul	20	ul	50	ul
yeast tRNA (20 mg/ml) (final 1 µg/ml)	0.5	ul	1	ul	2.5	ul
DEPC H2O	189.5	ul	379	ul	947.5	ul

  Do not vortex yeast tRNA solution.
  Dissolve and keep them at 60°C until use.

- Hybridization Mix B
  De-ionize Formamide:
  Add 4g mixed red resin to 40 ml Formamide in 50 ml Falcon tube and shake for 1h, pour formamide in new tube in such a way that red resin stays in old tube. SSC might not dissolve completely…

	for 10ml	**for 20ml**	**for 50ml**
Formamide (final 50%)	5.0ml	10ml	25ml
20xSSC (pH 7.0) (final 5x)	2.5ml	5.0ml	12.5ml
10% SDS (final 1%)	1.0ml	2.0ml	5.0ml
DEPC H2O	1.5mL	3.0mL	7.5ml

- Wash 1 (2xSSC)

	for 500mL
20xSSC (pH 7.0) (final 2xSSC)	50mL
10%CHAPS (final 0.1%)	5.0mL
H2O	up to 500mL

- Wash 2 (0.2xSSC)

	for 500mL
20xSSC (pH 7.0) (final 0.2xSSC)	5mL
10%CHAPS (final 0.1%)	5.0mL
H2O	up to 500mL

- AP-buffer

	for 10mL	**for 50mL**
1M Tris-HCl (pH 9.5) (final: 0.1M)	1.0mL	5.0mL
5M NaCl (final 0.1M)	0.2mL	1.0mL
10% TritonX-100 (final: 0.1%)	0.1mL	0.5mL
1M MgCl2 (final: 0.05M)	0.5mL	2.5mL
H2O	up to 10mL	50mL

  Comments:
  - Add 1M MgCl2 just before use to avoid pH change.

- Staining solution (NBT / BCIP)
  Check stock solution

	for 10mL
AP buffer	10mL
NBT (50mg/ml)	66µL
BCIP (50mg/ml)	33µL

  - Protect from light with foil.