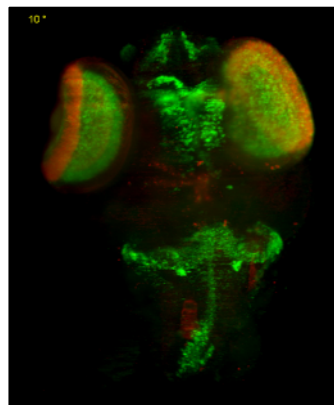


InsituPro UP protocol medaka whole mount 2

Date: 12.2007

System Configuration: Small baskets

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Atonal 5 specific probe (red, stained with Fast Red) and Pax6 specific probe (green, stained with TSA-Fluo), in a *Medaka* (*Oryzias latipes*) embryo head, stage 31 (Philipp Keller, Marcel Souren)

Description:

This protocol can be used for double fluorescent *in situ* hybridization on *Medaka* whole mounts. The method was established for the ***InsituPro*** upgrade, but can be easily adapted to the ***InsituPro VS*** or ***InsituPro VS*i****. Before using the ***InsituPro***, embryos are treated with Proteinase K in batches, according to their stage. Afterwards, embryos of different stages are pooled and pools of 12 specimens each processed with individual probes in the ***InsituPro*** robot. However, when using specimens of one stage for hybridization, rehydration and proteinase K treatment can be performed inside the instrument

Embryos are separated and fixed for 4h in 4%PFA/2xPTW. The chorion is removed and the embryos washed 4x 5 min in PTW. Finally, embryos are washed in 100% MeOH for 5 min and stored in 100% MeOH at -20°C at least over night.

Prior to incubation in the ***InsituPro***, Embryos are rehydrated for 5 min each in 75% MeOH/PTW, 50% MeOH/PTW, 25% MeOH/PTW and 2x in PTW. Digestion with Proteinase K was performed depending on the stage of the embryo.

Stage	Time of Proteinase K digestion
1-13	1-2 min
14-16	5 min
17	6 min
18	7 min
18-20	8-10 min
21-25	11-16 min
26	20 min
27-30	30 min
>30	>30 min

Stop Proteinase K by rinsing 2x in Glycine/PTW. Refix in 4%PFA/PBS for 20 min. Wash 5x 5min in PTW. Transfer to ***InsituPro*** and start run.

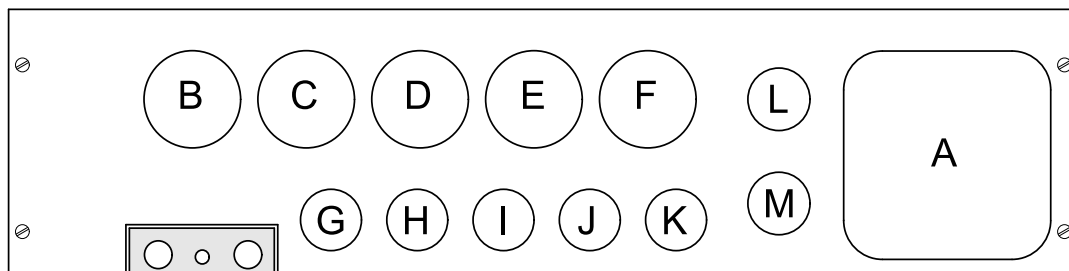
For staining, incubate embryos in TSA-Fluorescein solution for 45-60 min in the dark. All subsequent steps may be performed in the dark to avoid bleaching of stain. Wash 3x 5 min with PBS. Equilibrate in RSB. Add Fast Red staining solution and stain for up to 48h. Wash 3x 5min in PTW. Embryos can be kept in PTW for several days to remove background staining. For long term storage, embryos need to be kept in PTW/1% PFA in the dark.

The following protocol is designed for up to 30 whole mounts using the upgraded version of the **InsituPro**

Step No.	Task	Time	Action	Proceeding
1	PrimeNeedle		12000	
2	SetTempReg		T0 (OFF)	
3	IncubateVT	15 min	150 µl 1x PTW → Specimen	PTW wash
4	IncubateVT	15 min	150 µl Hyb-Mix → Specimen	Wash Hyb.-Mix
5	SetTempReg		T2 (HIGH)	65°C
6	IncubateVT	1 h	150 µl Hyb Mix → Slide	Prehybridization
7	IncubateVT	8 h	200 µl Probe → Specimen	Hybridization with Dig and Fluo labelled probe
8	IncubateVT	8 min	200 µl 2x SSCT/50%FA → Specimen 5x	Posthyb wash
9	IncubateVT	10 min	200 µl 2x SSCT/50%FA → Specimen	Posthyb wash
10	IncubateVT	8 min	200 µl 2x SSCT → Specimen 5x	Posthyb wash
11	IncubateVT	10 min	200 µl 2x SSCT → Specimen	Posthyb wash
12	IncubateVT	8 min	200 µl 2x SSCT → Specimen 5x	Posthyb wash
13	IncubateVT	10 min	200 µl 2x SSCT → Specimen	Posthyb wash
14	SetTempReg		T0 (OFF)	
15	Wait	30 min		Cool down
16	IncubateVT	10 min	150 µl PTW → Specimen 2x	PTW wash
17	IncubateTS	1 h	200 µl Blocking Solution → Specimen	Blocking
18	IncubateTS	6 h	200 µl antibodies → Specimen	DIG and FLUO
19	IncubateTS	8 min	200 µl PTW → Specimen 8x	PTW wash
20	IncubateTS	8 min	200 µl PBS → Specimen 2x	PBS wash
21	Pause		Take out embryos and perform staining	
22	SetTempReg		T0 (OFF)	
23	PrimeNeedle		12000	

Specimen and Buffer loading Form

Method: ISP UP *Medaka* whole mount 2 **User:** Marcel Souren **Date:** 03/12/2007



Buffer Loading:

Position	Buffer	Amount
A	PTW	
B	Hyb.-Mix	
C	2xSSCT/50% FA	
D	2xSSCT	
E	0.2xSSCT	
F	PBS	
G		

Position	Buffer	Amount
H	-	
I	-	
J	-	
K	-	
L	Blocking solution	
M	antibodies	
Probe		

Buffers:

Buffer : 10x PBS	pH : 7.5
Substance	Concentration
NaCl	137 mM
KCl	2.7 mM
Na ₂ HPO ₄ x 2H ₂ O	7 mM
KH ₂ PO ₄	1.5 mM

Buffer : PTW	
Substance	Concentration
1x PBS	
Tween 20	0.1% (v/v)

Buffer : 4% PFA	
Substance	Concentration
Paraformaldehyd in PTW	4% [v/v]

Buffer : Proteinase K	
Substance	Concentration
Proteinase K In 1x PTW	10 µg/ml

Buffer : Glycine/PTW	
Substance	Concentration
Glycine In 1x PTW	2mg/ml

Buffer : Hybridisation mix	
Substance	Concentration
Formamide	50% [v/v]
20x SSC	25% [v/v]
Heparin	150 µg/ml
tRNA	5 mg/ml
Tween 20	0.1% [v/v]

Buffer : 20x SSC	pH : 4,5
Substance	Concentration
NaCl	3 M
Di-Sodium Citrate Tri-hydrate	300 mM
adjust pH with citric acid	

Buffer : 2x SSCT/50% FA	pH: 7.5
Substance	Concentration
SSC	2x
Formamide (100%)	50% [v/v]
Tween 20	0,1%

Buffer : 2x SSCT	pH: 7.5
Substance	Concentration
SSC	2x
Tween 20	0,1%

Buffer : 0.2x SSCT	pH: 7.5
Substance	Concentration
SSC	0.2x
Tween 20	0,1%

Buffer : Blocking solution	pH 7.5
Substance	Concentration
Blocking reagent (Roche, Cat. No. 1 096 176) (10% (w/v))	40% [v/v]
Malate	200 mM
NaCl	300 mM
Tween 20	0.1%

Buffer : Antibody	pH 7.5
Substance	Concentration
Anti DIG-Alcaline Phosphatase	1:1000
Anti Fluo-Peroxidase	1:500
In Blocking solution	

Buffer : RSB	pH 8.2
Substance	Concentration
Tris/HCl	0.1 M
Tween 20	0.1%

Buffer : Fast Red RSB	pH 8.2
Substance	Concentration
RSB	2 ml
Fast red tablett	1

Buffer : TSA- Fluorescein	pH 7.5
Substance	Concentration
TSA-Fluorescein	1 part
TSA Amplification diluent	49 parts