

Paradise Fish Whole Mount *In Situ* Hybridization Protocol

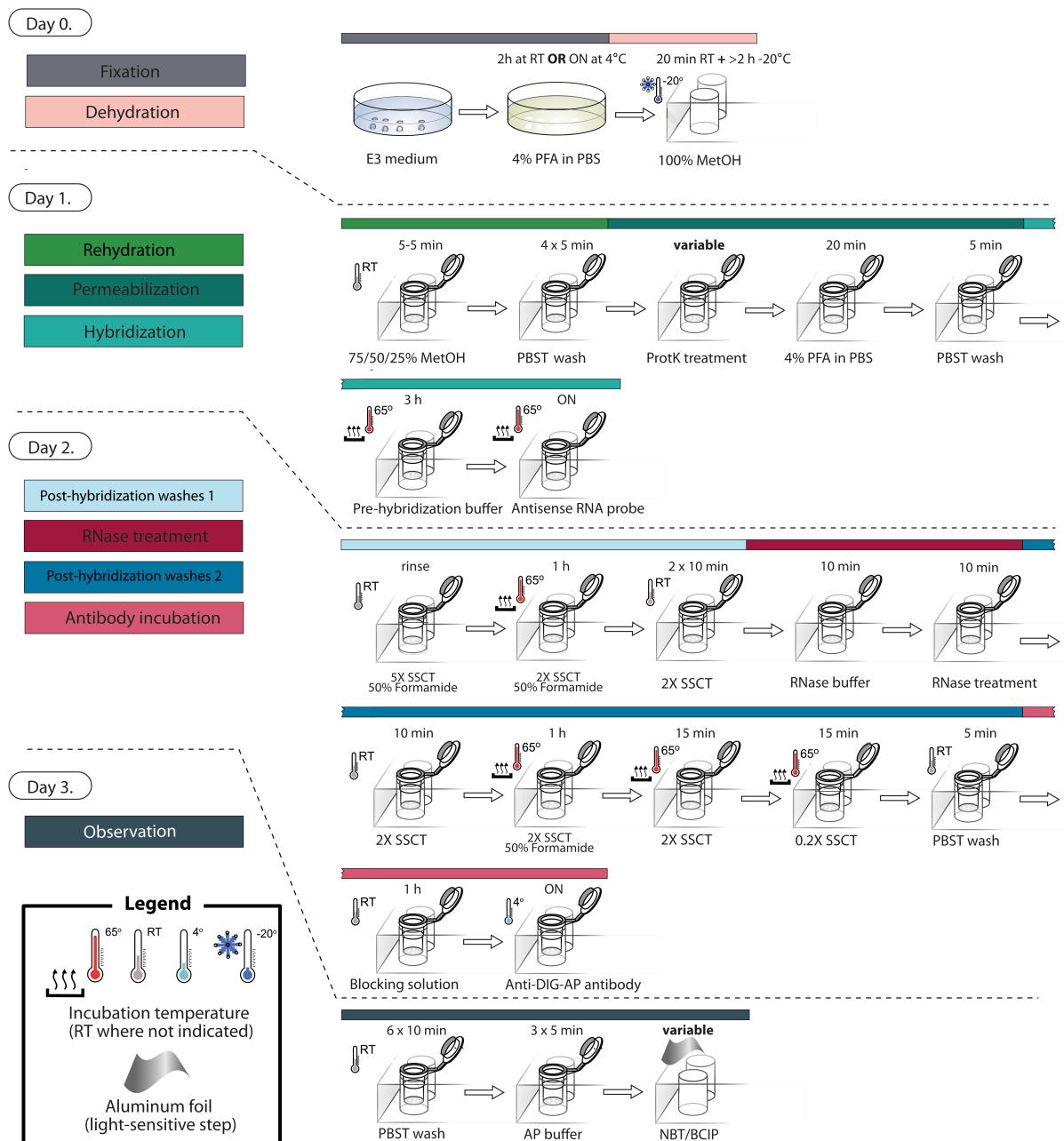
Szabó et al. (2025) Biologia Futura

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1 Graphical Overview of the Protocol



2 Materials and Small Equipment

Materials	Small Equipment
24-well plate	shaker
strainers (DIY)	waterbath
glass pipettes (wide bore)	
pasteur-pipettes (3 mL)	
parafilm	
aluminium foil	
glass container	

! Making DIY Stainers

We created our stainlers by gluing the upper part of 2 mL Eppendorf tubes to a piece of mosquito net with the mesh size of 0.2 x 0.4 mm.



(a) Plasticware used in this protocol.



(b) DIY stainlers.

Figure 1: Materials used in this protocol.

3 Solutions and Reagents

Recipes for Solutions

10× Phosphate Buffered Saline (PBS) Stock (1 L)

Component	Amount
NaCl	80 g
KCl	2 g
Na ₂ PO ₄	14.4 g

Component	Amount
KH ₂ PO ₄	2.4 g
Distilled water	Fill to 1 L

! 10x PBS Stock Note

Adjust pH to 7.4 and autoclave.

i Storage

Store at room temperature (RT).

PBS with Tween-20 (PBST) (50 mL)

Component	Amount	Final concentration
1× PBS	Fill to 50 mL	-
Tween-20 (20% stock)	250 µL	0.1%

💡 Tween-20 Tip

Tween-20 is highly viscous. Prepare a 20% Tween-20 stock solution (dilute pure Tween-20 1:5 in distilled water) for easier pipetting.

i Storage

Store at RT.

4% Paraformaldehyde (PFA) in PBS (50 mL)

Component	Amount
20% PFA powder	4 g
10× PBS	5 mL
Distilled water	Fill to 50 mL

⚠️ PFA is toxic, use it with caution!

Heat PFA solution in a fume hood to 60°C while stirring until dissolved.

i Storage

Long term storage at -20°C. After defrosting, aliquots can be stored at 4°C for a week.

Hybridization Buffer (50 mL)

Component	Amount
Formamide	25 mL
Tween-20 (20% stock)	250 µL
Yeast tRNA (50 µg/µL)	50 µL
Heparin (50 µg/µL)	50 µL
20× SSC	12.5 mL
Distilled water	Fill to 50 mL

i Storage

Store at -20°C.

20× Saline-Sodium Citrate (SSC) Solution (1 L stock)

Component	Amount
NaCl	175.3 g
Trisodium citrate	88.2 g
Distilled water	Fill to 1 L

i Storage

Store at RT.

5× SSC with Tween-20 (SSCT) / 50% Formamide (50 mL)

Component	Amount
Formamide	25 mL
20× SSC	12.5 mL
Tween-20 (20% stock)	250 µL
Distilled water	Fill to 50 mL

i Storage

Store at 4°C.

2× SSC / 50% Formamide (50 mL)

Component	Amount
Formamide	25 mL
20× SSC	5 mL
Tween-20 (20% stock)	250 µL
Distilled water	Fill to 50 mL

i Storage

Store at 4°C.

2× SSCT (50 mL)

Component	Amount
20× SSC	5 mL
Tween-20 (20% stock)	250 µL
Distilled water	Fill to 50 mL

i Storage

Store at 4°C.

0.2× SSCT (50 mL)

Component	Amount
20× SSC	0.5 mL
Tween-20 (20% stock)	250 µL
Distilled water	Fill to 50 mL

i Storage

Store at 4°C.

RNase Buffer

! RNase Buffer Note

Always prepare fresh RNase Buffer. Prepare double volume as it will be used for the RNase Solution as well.

Component	Amount
5 M NaCl	5 mL
1 M Tris-HCl (pH ~7.5-8)	500 µL
Tween-20 (20% stock)	250 µL
Distilled water	Fill to 50 mL

RNase Solution

! RNase Solution Note

Always prepare fresh RNase Solution.

Component	Amount
RNase A (20 µg/µL)	10 µL
RNase T1 (5 U/µL)	20 µL
RNase Buffer	10 mL

Blocking Solution

! Blocking Solution Note

Always prepare fresh Blocking Solution. Prepare double volume.

Component	Amount
10x PBS	5 mL
10% Blocking reagent	5 mL
Tween-20 (20% stock)	500 µL
Distilled water	Fill to 50 mL

10% Blocking Reagent (100 mL)

Component	Amount
Blocking Reagent	10 g
Maleic acid buffer	100 mL

! 10x Blocking Reagent Notes

Mix thoroughly while applying heat.

i Storage (10x Blocking Reagent)

Store at -20°C.

Maleic acid buffer (2 L)

Component	Amount
Maleic acid	23.2 g
NaCl	17.53 g
Distilled Water	Fill to 2 L

! Maleic Acid Notes

Dissolve components first in 1.5 L of Distilled Water. Adjust pH to 7.5 with concentrated or solid NaOH, sterile. Fill to 2 L.

i Storage (Maleic acid)

Store at RT.

Alkaline Phosphatase (AP) Buffer (10 mL)**! AP Buffer Note**

Always prepare fresh AP Buffer.

Component	Amount
1 M Tris-HCl (pH 9.5)	1 mL
1 M MgCl ₂	500 µL
5 M NaCl	200 µL
Tween-20 (20% stock)	50 µL
Distilled Water	Fill to 10 mL

Chromogen Solution (1 mL)

! Chromogen Solution Notes

Always prepare fresh Chromogen Solution. Use 500 µL per sample.

Component	Amount
NBT	4.5 µL
BCIP	3.5 µL
AP Buffer	1 mL

Reagents and Suppliers

Reagent	Supplier	Catalog.Number
Formamide	Sigma-Aldrich	75-12-7
Heparin	Sigma-Aldrich	H3393-50KU
Blocking solution	Roche	11096176001
Anti-Digoxigenin-AP, Fab fragments	Roche	11093274910
NBT	Roche	11383213001
BCIP	Roche	11383221001
RNase T1	Thermo Fisher	AM2280
RNase A	Thermo Fisher	EN0531
Torula yeast RNA	Sigma-Aldrich	R6625
Proteinase K	Thermo Fisher	E0049

⚠ Warning

These suppliers and catalog numbers are only indicative. Equivalent reagents from other suppliers will probably work equally well.

4 Step-by-Step Protocol

Day 0 - Fixation & Dehydration

3.1 Fixation

- Fix embryos in 4% PFA in PBS on shaker for 2 hours at RT **or** overnight (ON) at 4°C

3.2 Dehydration

Solution	Duration	Temperature
100% methanol (MetOH)	20 min	RT
100% MetOH	at least 2 h	-20°C

Day 1 - Rehydration, Permeabilization, Hybridization

Volumes Note

A volume of 1000 µL of the resepective solution is used at each step unless otherwise noted.

! Transfer embryos into strainers

Transfer embryos or samples to 24-well plates with strainers. This step is essential as due to the presence of the lipid droplet, embryos will float .

3.3 Rehydration

Step	Duration	Temperature
75% methanol / PBST	5 min	RT
50% methanol / PBST	5 min	RT
25% methanol / PBST	5 min	RT
PBST	4 × 5 min washes	RT

3.4 Permeabilization

i Proteinase K Digestion Notes

Duration depends on developmental stage. The following duration times are indicative - for the best results with your probe you should test multiple duration times.

Solution	Developmental stage	Duration
Proteinase K (1:1000 dilution in PBST)	Early epiboly	12 min
	Late epiboly	9 min
	Bud	9 min
	Somitogenesis	12 min

3.5 Post-fixation

Solution	Duration	Temperature
4% PFA in PBS	20 min	RT
PBST	5 min	RT

3.6 Hybridization

Solution	Duration	Temperature	Volume
Pre-hybridization Buffer	3 h	65°C	1000 µL
Probe in hybridization buffer (1:400 dilution)	ON	65°C	400 µL

Day 2 - Post-hybridization Washes, RNase Treatment, Antibody Staining

3.7 Post-hybridization Washes I

Solution	Duration	Temperature
5× SSCT / 50% formamide	Rinse briefly	RT
2× SSC / 50% formamide	1 h	65°C
2× SSCT	2 × 10 min	RT

3.8 RNase Treatment

Solution	Duration	Temperature
RNase buffer	10 min	RT
RNase solution	10 min	RT

3.9 Post-hybridization Washes II

Solution	Duration	Temperature
2× SSCT	10 min	RT
2× SSC / 50% formamide	1 h	65°C
2× SSCT	15 min	65°C
0.2× SSCT	15 min	65°C
PBST	5 min	RT

3.10 Blocking & Antibody Incubation

Solution	Duration	Temperature
Blocking Solution	1 h	RT
Anti-DIG-AP antibody (1:4000 dilution) in Blocking Solution	ON	4°C

Day 3 - Color Reaction & Observation

3.11 Post-antibody Washes

Solution	Duration	Temperature
PBST	6 × 10 min	RT
AP buffer	3 × 5 min	RT

💡 Pre-staining Tip

Transfer the samples to a 24-well plate, before applying the chromogen.

3.12 Chromogenic Reaction

Solution	Duration	Temperature
Chromogen Solution (AP buffer + NBT/BCIP)	≥ 30 min (ON)	RT (4°C)

! Chromogenic Reaction

The chromogen is light sensitive, therefore, **cover the plate with a foil** and place it on a slow moving shaker. Monitor color development regularly under a microscope. Length of development varies between probes. For strong probes usually 30 min is sufficient, but for most probes staining takes longer. If no (or faint) staining can be seen after 2 h, move the staining into 4°C for ON.

5 Stopping the Staining Reaction and Storage of Samples

i Long Term Storage Notes

Once the signal is strong enough, stop the staining as detailed below. PBST and 4% PFA washes can be performed in the plate, but move the samples into a labeled eppendorf tube before adding the 87% glycerol.

Solution	Duration	Temperature
PBST	3 × 5 min	RT
4% PFA in PBST	20 min	RT
PBST	5 min	RT
87% Glycerol	days - months	4°C

6 Results

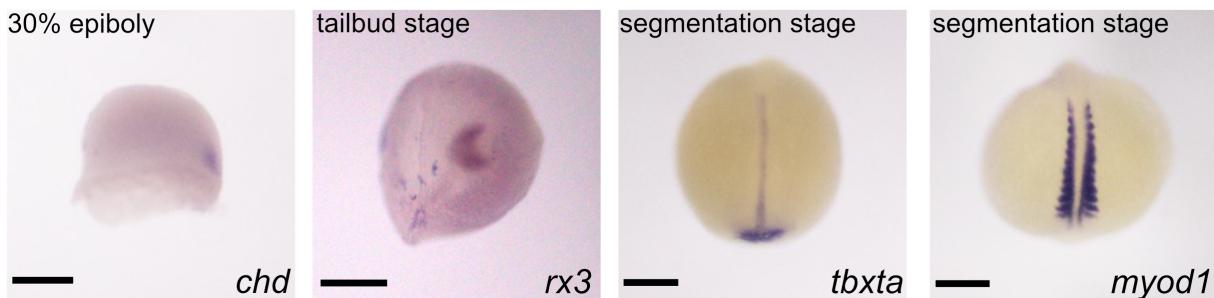


Figure 2: Typical examples for stained paradise fish samples of various stages.