### Wholemount Fluorescence ISH Protocol

### (Adapted from Rahul Parnaik-Ragsdale Lab by Gina Elsen and Gokhan Dalgin-Prince Lab University of Chicago)

**Embryos Fixation and Dehydration:**

1. Fix overnight at 4°C in 4% PFA.
2. Rinse 2X in PBT for 5 minutes
3. Dehydrate embryos through a series of methanol solutions at RT
	1. 30% MeOH in PBT for 5 minutes
	2. 60% MeOH in PBT for 5 minutes
	3. 100% MeOH in PBT for 5 minutes; replace with fresh 100% MeOH
4. Incubate dehydrated embryos at –20°C overnight (at this pointmay be stored up to 1year).

**DAY 1: Rehydration, ProK, Hybridization**

1. Rehydrate embryos through a series of methanol solutions at room temperature in 1.5ml tubes (optional: we usually transfer embryos in 48-well plate at this point till the end of the protocol)
	1. 60% MeOH in PBT for 5 minutes
	2. 30% MeOH in PBT for 5 minutes
	3. PBT for 5 minutes, 2X
	4. Wash once with 1XPBS 5 minutes
2. Incubated embryos on rocker in ***0.6% hydrogen peroxide in PBS for 10 minutes*** **at RT** (*you* *can increase H2O2 amount upto 2% but the embryos become too soft*) This step both bleaches the embryos and inactivates any residual peroxidase activity.
3. Wash 3X in **PBT**, 5 minutes each.

4. **Digest with 1X** (10μg/ml) Proteinase-K at RT (ProK is at 100X (1mg/ml) keep stock in -200C). For 1X, dilute 100μl into 10ml PBT

No somites no digetion

1-6 somites 3 minutes

7-12 somites 5 minutes

12-18 somites 10 minutes

18 somites -24hpf 13minutes

24hpf-36hpf 15minutes

48hpf 45 minutes

72hpf 1hour and 15 minutes

1. Post-fix embryos on rocker in **4% PFA for 1.5 hour at RT,**
2. Wash 4X in **PBT,** 5 minutes each, at RT.
3. Remove PBT and replace with prehybridization solution (**pre-HYB**)

**To Make Hybridization buffer (HYB-65%): In a 50 ml tube:**

Formamide (stored at -200C) 32.5 ml..........................…..65%

20 x SSC/DEPC 12.5 ml.............................5x

Heparin @ 50 mg/ml (40C) 50μl.......................……...50g/ml

20% Tween 250 μl...............................0.1%

0.5M Citric acid 920 μl...........................…to pH 6

DEPC/water **to 50 ml (store in –20°C)**

**Hybridization:**

1. Incubate embryos in 300l **pre-HYB** in **70°C incubator for 1-3 hours.**
2. Remove pre-HYB and add appropriate volume of the riboprobe(s) to the **HYB-tRNA (**Add 5mg tRNA to 10ml Hyb mix)**. Incubate in a 70°C incubator overnight (minimum 12 hours).**

**DAY 2: Post-hybridization Washes to Antibody Incubation:**

(Note: Pre-warm solutions A, B, C, 2X SSC, 0.05X SSC at **700C)**.

1. Remove probes (save the probes)
2. Washes, in **700C** incubator:

Wash A: 75% HYB + 25% 2X SSC, 10 minutes

Wash B: 50% HYB + 50% 2X SSC, 10 minutes

Wash B: 25% HYB + 75% 2X SSC, 10 minutes

2X SSC, 10 minutes

0.05X SSC, 30 minutes 2X

Note: Start thawing **Western Blocking reagent (WBR)** (#1921673, Roche) (Consider this as 10X stock and store in aliquots at -200C)

1. Washes at RT on a shaker:

Wash D: 75% 0.5X SSC + 25% PBT, 10 minutes

Wash E: 50% 0.5X SSC + 50% PBT, 10 minutes

Wash F: 25% 0.5X SSC + 75% PBT, 10 minutes

3X PBT, 5 minutes

1. Blocking step: incubate embryos in **1X WBR (dilute from the 10XWBR in PBT and filter it!!!) for 2 hours at RT.**

**5. Add anti-Dig-POD (**The reagent is an anti-digoxigenin antibody from sheep, Fab fragments, conjugated with polymerized horse-radish peroxidase [POD(p)]. **(Roche** 11 633 716 001**)** final concentration of 1:100-1000 in **filtered** 1X WBR. Incubate on rocker overnight at 4°C.

*Note:* Test appropriate antibody concentration so far 1:100-1000 range works for us.

All antibodies are POD (below there’s a mistake that it is HRP. This is wrong, tyramide is POD).

**DAY 3: Post-10Antibody Washes :**

1. Remove the anti-Dig-POD (HRP) 10 Antibody

2. Rinse 2X in PBT, and wash 6X in PBT, 10 minutes each

1. **Either:**
	1. Wash in PBT overnight at 4°C on rocker **OR**
	2. Proceed to tyramide reaction. **If time is not limiting, it is better to do the overnight wash to produce the cleanest possible results.**

**DAY 4: Tyramide reaction:**

1. Preincubate in AB (amplification buffer) for 10 minutes with rocking.

*Notes:*

* Use TSA Plus Cyanine3 System (Perkin Elmer NEL74400 1KT) to detect Dig-probes.
* Make TWS (tyramide working solution) just prior to the signal detection. To make TWS dilute the tyramide stock **1:100-1000** in 0.0015% H2O2/ amplification buffer (AB) Amplification buffer according to Vize is PBT with 10 mM Imidazole (Made from a 100 mM Imizadole stock) IS 100723. **Need about 200l of TWS /well**. Do serial dilution to make 0.0015% H2O2/AB: first make 0.15% H2O2 in AB (1:200 from 30% stock H2O2), then make AB/0.0015% H2O2 (1:100 from the 0.15% stock H2O2).

2. Incubate embryos on rocker in RT **for 1 hour in Cy3 Plus tyramide**, and protect from light.

3. Wash for 15 minutes in PBT, 3X in RT.

4. Can look at embryos on an epifluorescence or confocal microscope at this point or continue with detection of next hapten. Wash longer if high background.

**Second color reaction:**

**Begin protocol for 10 Antibody labeling for the 2nd probe:**

1. Incubate 10 minutes in 0.1 Glycine, pH2.2 (low pH in general cause the initial 10 antibody to be stripped away) (*Notes:* This step is optional)

2. Wash 5X in PBT

3. Block 2hours at RT in **1X WBR (filtered!!!)**

4. Incubate in anti-Fl-HRP 10 Antibody (Perkin Elmer NEF 710) (1:100-1000 in 1X WBR) overnight at 40C.

**DAY 5**: 2nd TSA reaction

1. Remove 10 Antibody (for the 2nd color reaction)

2. Rinse 2X in PBT, and wash 6X in PBT, 10 minutes each (Notes: Wash longer or overnight if possible)

3. Incubate in Amplification Buffer (we use home brew amplification buffer) for 15 minutes at RT.

(Notes: Make Tyramide Working Solution (TWS) just prior to the signal detection. To make TWS dilute the tyramide stock 1:100-1000 in 0.0015% H2O2/ amplification buffer (AB). Need about 200μl of TWS /well. Do serial dilution to make 0.0015% H2O2/AB: first make 0.15% H2O2 in AB (1:200 from 30% stock H2O2), then make AB/0.0015% H2O2 (1:100 from the 0.15% stock H2O2).

1. Incubate for 1 hour in FITC-TSA (Perkin Elmer). (We use home-made Fluorescein-tyramide diluted in home brew amplification).
2. Wash 3 X for 15 minutes (or longer and observe color reaction).

## Solutions and Reagents

**Home Brew: For 1 L**

 To 1 L of DEPC-treated PBS add

 0.1 M Imidazole 6.8g Imidazole

**Fluorescein Tyramide**

**Adapted from Vize et. al., NATURE PROTOCOLS**

**VOL.4 NO.6  2009  p975 and Rahul Parnaik-Ragsdale Lab by Gokhan Dalgin**

Basic idea: react reactive succinimidyl ester with tyramine hydrochloride under

anhydrous conditions and with correct stoichiometry.

So, use fresh (anhydrous) DMF.

1) Make FL-ester solution at 10mg/ml in DMF:

100mg NHS-fluorescein (MW=473, NHS-Fl is moisture-sensitive equilibrate vial to RT before opening **read manufacturers product information!!!**) added to 10ml DMF

2) Make DMF-TEA solution:

5ml DMF

50ul TEA

3) Make reactive tyramine solution:

50mg tyramine hydrochloride added to 5ml TEA-DMF solution.

4) Mix reagents:

10ml FL-ester solution

3.425ml tyramine solution

React for 2hr at room temp in the dark (ie, cover with foil or place in a drawer)

Then add 11.5 ml 100% ethanol. This makes a 100x stock.

Make 1ml aliquots. It is good for about a year in -20 or -80

**Just to point out the cost diff between various dyes:**

**100mg Fluorescein Ester = $110**

**1mg Cy3/Cy5 ester = $214**

**Reagents**

DMF Anhydrous, Acros CAS: 68-12-2, Product Code 610941000

Triethylamine 99% prue, Acros CAS:121-44-8, Product Code 157911000

Tyramine hydrochloride 99% Sigma CAS: 60-19-5 Product Number T2879 1G

Ethanol 200 proof Sigma CAS: 64-17-5 Product Number E7023

NHS-Fluorescein, 100mg Thermo Scientific Product number 46410

Cy3 Mono NHS Ester GE healthcare/Amersham PA13101