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Stickleback *(Gasterosteus aculeatus)* Whole-Mount Basket In Situ Hybridization
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Introduction

This guide describes how to visualize gene expression in whole or partial stickleback embryonic, larval, or adult tissue. To reduce damage to delicate tissues during wash cycles, samples are placed in mesh baskets and moved from well to well of a tissue culture plate, with each well containing the liquid for each wash cycle. This protocol is based largely on the Thisse protocol for in situ hybridization on zebrafish embryos (Nature Protocols, 3: 59-69, 2008).

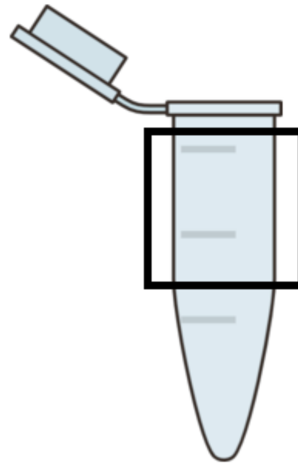
Set-up

Baskets

Materials

- 1.5 mL epitubes
- 100 micron Nitex Nylon filters with 1-2” diameter
  (e.g. Sefar #B000DN1DP8)

Cut off the cap, top lip, and bottom of the epitube with a hot razor blade to obtain an open plastic cylinder, as shown in the right image.



Discard all regions of the epitube outside the box.

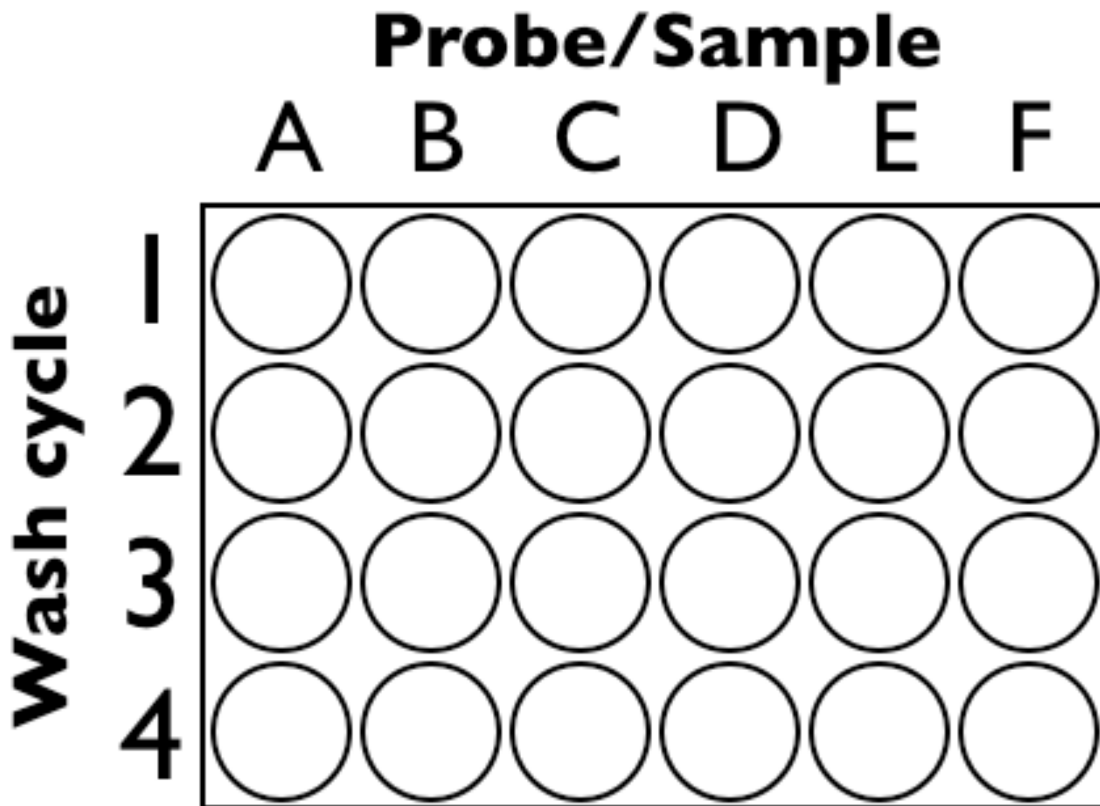
Cover a hot plate with aluminum foil and heat at a low setting (usually around setting # 2) **The hotplate should be cool enough that the nylon will not melt.**

Place the nylon filter on the foil and press the epitube firmly against the plate, checking to make sure that the plastic has created a firm seal on the nylon by melting into it. Cool by immersing in iced MilliQ water.

Sterilize the basket by soaking in 0.1 M NaOH for 1 hr to overnight. Rinse 3-5 times in MilliQ water. Rinse once in methanol. Store in fresh methanol until next use. Baskets may be reused until they start falling apart.

Plates

Tissue culture plates (e.g. BD Falcon 353047 Multiwell 24-well Flat Bottom with Low Evaporation Lid) work well as vessels for most of the washes in this protocol. In this guide, **washes that should be done in a tissue culture plate are given in a table**. To wash the sample, move the mesh basket from one well to another using RNAse-free forceps. It is often easiest to have one column for each sample, and one column for each wash cycle, as shown in the diagram below:



Most plates can be reused indefinitely. Sterilize plates by soaking overnight in 0.1M NaOH and rinsing with MilliQ water. To reduce cross-contamination, use only one kind of solution in each plate. **Always throw out plates used for riboprobe hybridization steps.**

Procedure
WARNING: RNAse contamination can degrade the quality of your in situs. Be as sterile as possible, especially on Days 1 and 2 of this procedure.

Day 1: Rehydration, dissection, proteinase K digestion, probe hybridization
A. Rehydration
Rehydration of tissue should be done in epitubes. Begin with tissue that has already been fixed in formaldehyde and stored in 100% methanol.

Wash the tissues in 1 ml of 66% Methanol, 33%PBST solution for five minutes.

Wash the tissues in 1 ml of 33% Methanol, 66%PBST solution for five minutes.

Wash the tissues in 1 ml of 100% PBST for five minutes. Repeat.

B. Dissection
Dissections can be performed in small petri dishes containing PBST. Ensure that the tissue does not dry out during this step.

Embryos that have not yet hatched (<8dpf) should be de-chorionated to increase the penetration of the probe, as well as de-yolked. If desired, remove the head and eyes of >15dpf fish to allow the probe to better penetrate the tissues in the head.

C. Bleach
Prior to bleaching, transfer tissue to a white spotting plate. This will make the cleared tissue more visible and less likely to be lost before being transferred to the tissue culture plates. **Thisse bleach should always be made fresh. If gas does not evolve from the solution ("fizz"), the bleach will not work.** Because hydrogen peroxide loses activity over time, buy hydrogen peroxide in the smallest quantity possible, and be careful to monitor the age of the hydrogen peroxide and how effectively it bleaches melanin. *Note that bleaching times may need to be extended if you are using older hydrogen peroxide.*

Immerse the tissue in Thisse bleach solution for 5-10 minutes. **Do not over-bleach, as this can damage cells.**

Preheat incubator to 68 C. Transfer tissue to baskets, and do the following washes in tissue culture plates:

Wash type	Time	Temperature	Volume	Cycles
1. PBST	5 min	Room Temp	1 mL	x3

Wash type	Time	Temperature	Volume	Cycles
2. 20 ug/mL ProK in PBST, 1% DMSO	10 min	RT	1 mL	x1
3. PBST	5 min	RT	1 mL	x3
4. 4% PFA, 1% DMSO in 1x PBS	20 min	RT	1 mL	x1
5. PBST	5 min	RT	1 mL	x4
6. Pre-hybridization solution	1 hr	68 C	1 mL	x1
7. Hybridization solution	>24 hr	68 C	500 uL	x1

Note: ProK digestion periods vary based on the thickness and location of the tissue of study. Incubation times need to be determined empirically for later stages or dissected-out tissues.

Ensure that the tissue is completely immersed in hybridization solution. Seal the plate securely with PCR film to reduce evaporation and the possibility of cross-contamination between probes. Leave to hybridize for at least 24 hours. For probes of more lowly expressed genes, and for larger pieces of larval or adult tissue, a two-day hybridization is recommended.

Day 2: Continue probe hybridization.
Allow the probe to continue hybridizing, to improve the quality of stain.

Day 3: Wash off probe, block and antibody
A. Washing off the probe
Minimize the time the tissue spends outside of the incubator. If the tissue cools, the stain may be nonspecific. Each plate should be preheated in the incubator for 10 minutes prior to transferring tissue to it.

The 2:1 and 1:2 prehyb/2x SSC solutions should be made fresh.

Discard the plate that contained hybridization solution to avoid cross-contamination in future experiments.

Wash type	Time	Temperature	Volume	Cycles
1. 66% prehyb, 33% 2x SSC	0,10 min	68 C	1 mL	x2
2. 33% prehyb, 66% 2x SSC	20 min	68 C	1 mL	x1
3.2x SSC	5 min	68 C	1 mL	x1

Wash type	Time	Temperature	Volume	Cycles
4. 0.2x SSC	30 min	68 C	1 mL	x2
TRANSFER TO ROOM TEMP.				
4. 66% 0.2X SSC, 33% PBST rinse	0 min	RT	1 mL	x1
5. 33% 0.2X SSC, 66% PBST rinse	0 min	RT	1 mL	x1
6. PBST rinse	0 min	RT	1 mL	x2

B. Block and antibody
Anti-digoxygenin antibody should be diluted 1:5000 in block.

Wash type	Time	Temperature	Volume	Cycles
7. Block	1 hr	Room Temp	700 mL	x1
8. Block and anti-dig. antibody	>12 hr	4 C	700 uL	x1

Seal the plate in PCR film to reduce evaporation, as high concentration of antibody may result in nonspecific staining.

Day 4:
Color solution should be made fresh. Pipet tips can now be reused between wells.

A. Washing off antibody:

Wash type	Time	Temperature	Volume	Cycles
1. PBST rinse	0 min	Room Temp	1 mL	x2
2. PBST wash 1	15 min	RT	1 mL	x3
3. PBST wash 2	15 min	RT	1 mL	x3

Transfer tissue to white spotting plates.

Immerse tissue in 500 uL of color solution for 5 minutes. Repeat this step twice.

Develop the stain in 500 uL of staining solution until desired depth of stain is reached. Keep the tissues in the dark during this process to ensure that the stain does not degrade. **Do not view developing tissues under bright lights, as this will increase nonspecific staining.**

Stop the developing reaction by rinsing the tissues **twice** in MilliQ water. Then, do **six** 10-minute washes in PBST. This series of rinses helps to remove as much staining solution from the tissue as possible. **If the tissue is not washed thoroughly, the reaction will continue to run and the tissues will stain completely dark in storage.**

To ensure that the staining reaction stops completely, transfer the tissues to 1.5 mL microtubes and immerse in 4% PFA in 1x PBS for at least 20 minutes to overnight at 4 C.

Prior to imaging the tissues, clear nonspecific staining by moving tissue through a series of 3-minute glycerol washes: 30% glycerol:70% PBST, 50% glycerol: 50% PBST, 70% glycerol: 30%PBST, and finally 100% glycerol. Tissues can then be mounted on slides, or stored in fresh 100% glycerol at 4 C.

Solutions | Final concentrations

Thisse Bleach

Makes 5 mL.	
2.5 mL 1% KOH	0.5% KOH
0.5 mL 30% H_2O_2	5 H_2O_2
2 mL milliQ water	*Make fresh for each experiment.*

Prehybridization solution

Makes 50 mL.	
25 mL formamide	50% formamide
12.5 mL 20x SSC	5x SSC
250 uL 20% Tween-20	0.1% Tween-20
460 uL 1M citric acid	pH 6.0
to 50 mL with MilliQ water	*Store at -20 C.*

Hybridization solution

Makes 50 mL.	
25 mL formamide	50% formamide
12.5 mL 20x SSC	5x SSC
250 uL 20% Tween-20	0.1% Tween-20
50 uL 50 mg/mL heparin	50 ug/ml heparin
500 ul 50 mg/mL tRNA	500 ug/ml tRNA
460 ul 1M citric acid	pH 6.0
to 50 mL with MilliQ water	*Store at -20 C.*

Block solution

Makes 50 mL.	
5 mL 10x PBS	1x PBS
250 uL 20% Tween-20	0.1% Tween-20
1 mL sheep serum	2% sheep serum
0.1 g BSA	2 mg/mL BSA
to 50 mL with MilliQ water	*Store @ -20; can thaw/refreeze*

Color solution

Makes 50 mL.	
5 mL 1 M Tris, pH 9.5	100 mM Tris pH 9.5
2.5 mL 1 M $MgCl_2$	50 mM $MgCl_2$
1 mL 5M NaCl	0.1M NaCl
250 uL 20% Tween-20	0.1% Tween-20
to 50 mL with MilliQ water	*Make fresh for each experiment.*

Staining solution

Per 1 mL color solution, add 4.5 uL NBT	0.45% NBT
and 3.5 uL BCIP.	0.35% BCIP
	Make fresh for each experiment.