Semi-Automated Whole Mount in situ Hybridisation

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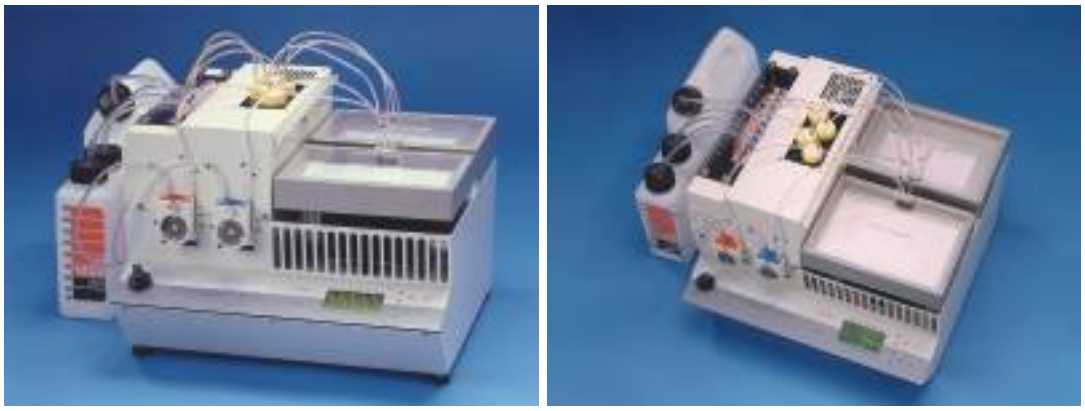
Using the BioLane HTI Robot

The following method is partially based on the protocol by Wilkinson & Nieto *Methods Enzymol.* 1993;225:361-73 and adapted by Gemma Martinez and Brooke Gardiner.

BioLane HTI Robot



The BioLane HTI robot consists of two systems, a blue and a red system. Each system has 9 plastic tubes hooked into the robot labeled 1 to 9. The robot has been programmed so each tube has been assigned to a specific solution. By inserting the tube into the solution the robot will automatically take the required amount as generated by a script. The robot will gently rock the samples in a tray while the program is in progress.

Nylon mesh baskets are used by the robot to hold the tissues, each basket represents one probe. Three sizes tray are available with the BioLane HTI robot, each holds a different number of nylon mesh baskets. We routinely use the small (20 basket) tray.

Pretreatment of Embryos

1. Dissect embryos of 9.5dpc (whole embryo), 10.5dpc (forelimb-hindlimb) and 12.5dpc(urogenital tract) in ice-cold PBS.
2. Immediately fix the tissues with 4% paraformaldehyde (PFA) overnight (16-18hours) at 4°C.

3. Wash the tissues twice with PBTX for 10 minutes each at room temperature.
4. Dehydrate with a series of 25% MeOH/75%PBTX, 50%MeOH/50%PBTX, 75% MeOH/25%PBTX washes for 20 minutes each, follow by two washes of 100% MeOH for 20minutes.
5. Store embryo tissues in 100%MeOH at -20°C.

Day 1- Cleaning, Re-hydration and Hybridisation (Blue System)

1. Place tray with nylon mesh baskets into the blue system and run the cleaning program.
2. Wash all the tubing in the blue system with 0.2M NaOH. (This is to maintain an RNase-free environment)
3. Wash all the tubing in the blue system with RO-H_2O.
4. Carefully put the embryonic tissues into the baskets. Each basket contains 2x 9.5dpc, 3x 10.5dpc, 1 male and 1 female 12.5dpc, and 2 x12.5dpc/2d explants.
5. Start the re-hydration program of the blue system.
6. The samples are treated as follows:

Re-hydration	RT	5 Minutes	100% MeOH
	RT	5 Minutes	75% MeOH/ 25% PBTX
	RT	5 Minutes	50% MeOH/ 50% PBTX
	RT	5 Minutes	25% MeOH/ 75% PBTX
	RT	10 Minutes	PBTX
	RT	5 Minutes	PBT
	RT	60 Minutes	PBT/ 6% H2O2
	RT	5 Minutes	PBT
	RT	5 Minutes	PBTX

	RT	5 Minutes	PBTX
Digestion	RT	20 Minutes	10ug/ml Proteinase K/ PBTX
	RT	5 Minutes	PBTX
	RT	5 Minutes	PBTX

7. Turn off robot and wash samples with 0.2% gluteraldehyde/4% PFA in PBTX in the fume hood at room temperature for 20 minutes.
8. Wash twice with PBTX at room temperature for 10 minutes each.
9. Remove baskets from tray and place baskets into a 48 well Cellstar plate containing 0.5ml pre-hybridisation solution in each well at 65°C for 2 hours.
10. Transfer baskets to a 48 well Cellstar plate with 0.5ml hybridisation solution at 65°C overnight. (Hybridisation solution = pre-hybridisation solution + ~0.2µg/ml DIG-labelled RNA probe.)

Preparation

11. Prepare the post-hybridisation washes and place at 65°C overnight.

Preabsorption of anti-DIG antibody

12. Prior to starting post-hybridisation washing, prepare the pre-blocking solution and pre-absorb the anti-DIG antibody Roche11093274910. (The pre-absorbed anti-DIG antibody can be kept at 4°C and recycled up to 3 times.)
18mg of embryo powder is placed in a 10ml tube with 10% sheep serum, 2% BSA in TBTX and 15µl anti-DIG antibody.
Incubate at 4°C for 3 hours or longer with gentle rocking.
Spin sample in a microfuge for 10 minutes, 4°C at 13000rpm.
Collect the supernatant and dilute it with 10% sheep serum, 2% BSA in TBTX.
Store the antibody at 4°C until use.

DAY 2 – Post-Hybridisation and pre-blocking (Red system)

1. Remove the baskets from the 48 well plate and place in a tray filled with solution 1.
2. The robot will preheat the rocker with the samples to 65°C for 15 minutes.
3. The following stringency washes are performed:

Post-Hybridisation	65°C	5 Minutes	100% Solution 1
washes	65°C	5 Minutes	75% Solution1/25% 2x SSC
	65°C	5 Minutes	50% Solution 1/50% 2x SSC
	65°C	5 Minutes	25% Solution 1/75% 2x SSC
	65°C	10 Minutes	2x SSC / 0.1% CHAPS
	65°C	10 Minutes	2x SSC / 0.1% CHAPS
	65°C	5 Minutes	0.2x SSC / 0.1% CHAPS
	65°C	5 Minutes	0.2x SSC / 0.1% CHAPS
	RT	10 Minutes	TBTX
	RT	10 Minutes	TBTX
Pre-blocking	RT	2 Hours	Pre-Block Solution
	4°C	Overnight	Pre-absorb anti-DIG antibody

Preparation

4. Prepare 0.1% BSA / TBTX at the end of day 2 and insert tubing into solution. The robot will start taking in 0.1% BSA / TBTX early morning of day 3.

Day 3 – Post-antibody washes and Development

1. The samples are washed as follows:

Post-Antibody	RT	30 Minutes	0.1% BSA / TBTX
washes	RT	30 Minutes	0.1% BSA / TBTX
	RT	30 Minutes	0.1% BSA / TBTX
	RT	30 Minutes	0.1% BSA / TBTX
	RT	30 Minutes	0.1% BSA / TBTX
	RT	10 Minutes	TBTX
The pH for NTMT decreases over time so it needs to be made fresh. The robot will pause for you to make up fresh NTMT and re-start it			
	RT	10 Minutes	TBTX
	RT	10 Minutes	NTMT
	RT	10 Minutes	NTMT
	RT	10 Minutes	NTMT

2. Transfer contents of the baskets into 12 well plates with 1ml of BM purple substrate Roche11442074001 in each well.
Bring the BM purple AP substrate to 15°C –20°C before using, no dilution is required but you may need to filter the solution to remove any precipitate.

3. Wrap the 12 well plates with aluminium foil and monitor samples for colour development approximately every 15-30 minutes.
Note: Normally 3 control samples will be used to verify the success of the run. Controls are Wnt4 (strong), Wnt7b (medium) and Shh (weak). For Wnt4 and Wnt7b, generally the expression develops within 1 to1.5hours whereas Shh can take 25-30 hours.

4. When the colour has proceeded to the desired extent, stop the colour development by transferring the samples to a new 12 well plate containing distilled water. Depending on the extent of colour development, samples can be left overnight to continue developing or kept at 4°C to slow down the reaction.
Note: If the samples have been over-developed, wash several times in PBS with 1% Triton X-100. This can be done for several days if necessary and will decrease the background issue.

5. Wash 3x with PBS at room temperature for 5 minutes each.

6. Fix samples with cold 4% paraformaldehyde at room temperature for 30 minutes.

7. Wash 3x with PBS at room temperature for 5 minutes each.

8. Store samples in 2ml Eppendorf tubes with PBS at 4°C.

9. Photograph as soon as possible. Use a petri dish with 1% agarose as a background.

Solutions & Volumes Required For W-ISH Using The Biolane Hti Robot

Small Tray (20 Baskets)

DAY1

Methanol 70mL
PBTX 350mL
PBT 80mL
PBT/H_2O_2 50mL
Proteinase K 50mL
Glut/PFA/PBTX 40mL

PBT/H_2O_2
10mL H_2O_2
40mL PBT

Proteinase K (Roche3115879)
25uL 20mg/mL stock proteinase K
50mL PBTX

Glut/PFA/PBTX
320uL 25% glutaraldehyde

48uL Triton X-100
40mL 4% PFA in PBS

DAY2

Pre-Block 50mL
Antibody 50mL
Solution1 70mL
2xSSC 70mL
2xSSC+CHAPS 80mL
0.2XSSC+CHAPS 80mL
TBTX 180mL
TBTX+0.1%BSA 200mL
NTMT 120mL

Pre-Block (100mL) N.B. If re-using antibody only require 50mL

2g BSA
10mL Sheep Serum
90mL TBTX

Antibody – Can store in fridge and re-use up to 3 times

15uL anti-DIG antibody (Roche11093274910)
3mL Pre-Block
18mg Embryo powder

Incubate this solution on rocker in cold room for 3 hours minimum and then spin down at 13 000 rpm for 10min. Collect the supernatant and add to 50mL pre-block solution before using in the *in situ* hybridisation.

Solution 1

50mL Formamide
25mL 20x SSC
100uL Triton X-100
10mL 5% CHAPS
15mL water

2xSSC+CHAPS (80mL)	**2xSSC+CHAPS (100mL)**
8mL 20xSSC	10mL 20xSSC
1.6mL 5% CHAPS	2mL 5% CHAPS
70.4mL H_2O	88mL H_2O
0.2xSSC+CHAPS (80mL)	**0.2xSSC+CHAPS (100mL)**
0.8mL 20x SSC	1mL 20x SSC
1.6mL 5% CHAPS	2mL 5% CHAPS
77.6mL H_2O	97mL H_2O

TBTX+0.1%BSA

0.2g BSA

200mL TBTX

NTMT

12mL 1M NaCl
12mL 1M Tris.Cl pH9.5
6mL 1M $MgCl_2$
120uL Tween 20
90mL H_2O

Colour development solution

BM Purple (Roche 11442074001)

Solution Preparation for the W-ISH		
PBTX		
Volume	Component	Final Concentration
50µl	Triton X-100	0.10%
50ml	1x PBS	
50ml	Total volume	
TBTX		
Volume	Component	Final Concentration
12.5ml	1M Tris.HCl pH 7.5	50mM
37.5ml	1M NaCl	150mM
250µl	Triton X-100	0.10%

199.75ml	Nuclease free H2O	
250ml	Total volume	
NTMT (prepare fresh when needed-pH decrease over time)		
Volume	Component	Final Concentration
5ml	1M NaCl	100mM
5ml	1M Tris HCl pH9.5	100mM
2.5ml	1M MgCl2	50mM
50µl	100% Tween 20	0.10%
37.45ml	Nuclease free H2O	
50ml	Total volume	
Solution 1		
Volume	Component	Final Concentration
25ml	Formamide	50%
12.5ml	20x SSC pH5	5x

50µl	Triton X-100	0.10%
5ml	CHAPS	0.50%
7.45ml	Nuclease free H2O	
50ml	Total volume	

2x SSC/ 0.1% CHAPS			
Volume	Component	Final Concentration	
5ml	20x SSC pH5	2x	
1ml	5% CHAPS	0.10%	
44ml	Nuclease free H2O		
50ml	Total volume		

0.2x SSC/ 0.1% CHAPS		
Volume	Component	Final Concentration
0.5ml	20x SSC pH5	0.2x
1ml	5% CHAPS	0.10%
48.5ml	Nuclease free H2O	
50ml	Total volume	

in situ pre-block		
Volume	Component	Final Concentration
1ml	100% Sheep serum	10%
0.2g	BSA powder	2%
~8.8ml	TBTX	
10ml	Total volume	
PBT		
Volume	Component	Final Concentration
2ml	Tween 20	1%
200ml	PBS	
5% CHAPS		
Volume	Component	Final Concentration
2.5g	CHAPS	5%

50ml	Nuclease free H2O	
4% Paraformaldehyde		
Volume	Component	Final Concentration
2g	Paraformaldehyde	4%
50ml	1x PBS	

20x SSC		
Volume	Component	Final Concentration
87.65g	NaCl	3M
44.1g	Na Citrate	0.3M
to 400ml	Nuclease free H2O	
pH to .0	10M NaOH	
to total	Nuclease free H2O	
500ml	Total volume	
Pre-Hybridisation Solution		

Volume	Component	Final Concentration
50ml	Formamide	50%
25ml	20x SSC	5x
2g	Blocking powder	2%
100µl	Triton X-100	0.10%
10ml	5% CHAPS	0.50%
100mg	Torula Yeast RNA	1mg/ml
1ml	0.5M EDTA	5mM
5mg	Heparin	50ug/ml
to total	Nuclease free H2O	
100ml	Total volume	
* Torula Yeast RNA can be added straight to the solution		
1M EDTA		
Volume	Component	Final Concentration
93.05g	EDTA	1M

to 200ml	Nuclease free H2O	
pH to 8	10M NaOH	
to total	Nuclease free H2O	
250ml	Total volume	

0.5M EDTA		
Volume	Component	Final Concentration
93.05g	EDTA	0.5M
to 400ml	Nuclease free H2O	
pH to 8	10M NaOH	
to total	Nuclease free H2O	
500ml	Total volume	
1M Tris		
Volume	Component	Final Concentration
121.1g	Tris-base	1M
to 800ml	Nuclease free H2O	

pH	Concentrated HCl	
to total	Nuclease free H2O	
1000ml	Total volume	
* Approximate Volumes for pH correction for 1000ml Tris		
70ml	Concentrated HCl	pH 7.4
60ml	Concentrated HCl	pH 7.6
42ml	Concentrated HCl	pH 8.0
If the 1M solution has a yellow colour, discard it and obtain better quality Tris		
Mouse Embryo Powder		
~12.5 - 14.5 dpc mouse embryos homogenized in minimal volume PBS		
+ 4x volume acetone ($0^{o}C$)		
$0^{o}C$ at 30 minutes		
Spin 10 000g 10 minutes		
Remove and discard supernatant		

+ acetone (0^{o}C)		
Spin 10 000g 10 minutes		
Remove and discard supernatant		
Spread pellet out and grind to fine powder on a sheet of filter paper		
Allow to air dry		
4 ^{o}C at storage in airtight container		

Associated publications

Rumballe BA, Chiu HS, Georgas KM and Little MH.
Use of in situ hybridization to examine gene expression in the embryonic, neonatal and adult urogenital system.
Methods in Molecular Biology: Kidney Development (commissioned book chapter).

Rumballe B, Georgas K, Little MH.
High-throughput paraffin section in situ hybridisation and dual immunohistochemistry
CSH Protocols, 2008

Georgas K, Rumballe B, Wilkinson L, Chiu HS, Lesieur E, Gilbert T, Little MH.
Use of dual section mRNA in situ hybridisation/immunohistochemistry to clarify gene expression patterns during the early stages of nephron development in the embryo and in the mature nephron of the adult mouse kidney.
Histochem Cell Biol. 2008 Nov;130(5):927-42. Epub 2008 Jul 11.