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## **Staff**

John Macauley, Director of Courses and Conferences; The Jackson Laboratory (JM)  
William Dove, co-director; Wisconsin (WD)  
David Threadgill, co-director; North Carolina (DT)  
Robert Whitehead, Vanderbilt (RW)  
Jeff Franklin, Vanderbilt (JF)  
Janan Eppig, The Jackson Laboratory (JE)  
Richard Halberg, Wisconsin (RH)  
Xiaodi Chen, Wisconsin (XC)  
Kevin Haigis, MIT (KH)  
Erica Rinella, North Carolina (ER)  
Bruce Aronow, Cincinnati (BA)

### Cameo appearance Thursday morning

Inke N  thke, Dundee, Scotland (IN)  
Maaike van der Born, Utrecht, Holland (MB)

## **Course members**

### Team assignment

Jim Amos-Landgraf, University of Wisconsin-Madison	G1
Diana Borenshtein, MIT	T1
Ian Carroll, University of North Carolina-Chapel Hill	G2
Dan Carson, University of Cincinnati	T1
Jessica Chen, University of California-Irvine	R1
Ann Cheung, MIT	G1
Flavia Girolani, Torino, Italy	G2
James Harper, Bristol, UK	R1
Takanori Kitamura, Kyoto, Japan	R2
Larry Kwong, University of Wisconsin-Madison	R2
Maria Larriba, Madrid, Spain	T2
Lisa McPhatter, Duke University	G2
Paloma Ord��nez, Madrid, Spain	R1
Oliver Sieber, Cancer UK-London	T2
Mark Sinnamon, Vanderbilt University	G1
Kevin Spring, Brisbane, Australia	T1
Ming Yu, University of North Carolina-Chapel Hill	R2

## Schedule

### *Sunday, September 26*

Arrivals and room check - in at Highseas Conference Center, Schooner Head Road

4:30-7:00 pm Social time and buffet dinner at Highseas

7:00 pm Orientation session with the course directors William Dove and David Threadgill, along with Bob Whitehead, Jeff Franklin, Rich Halberg, Xiaodi Chen, Kevin Haigis, Erica Rinella, and John Macauley, Highseas lecture room

The group of 17 students will be divided into three teams: Greeks (G), Romans (R), and Trojans (T). For some modules, a team will be further subdivided into 2 or 3 course members – e.g., Roman 1 and Roman 2.

Rotation:

Module	Monday	Tuesday	Wednesday
1	G	T	R
2	R	G	T
3	T	R	G

### *Monday, September 27*

7:00am Breakfast at Highseas

7:50am Shuttles to lab

8:00am Training Lab Orientation

8:30am-5pm Modules; in three teams, members will rotate over the course of the workshop amongst:

- Mouse biology and germline genetics (Module 1)
- Cellular and tissue biology (Module 2)
- Molecular genetics (Module 3)

On Monday, lunch will be provided so that members and staff will be able to break for lunch at their own pace, in the nearby conference room (GRB3225).

6:00pm Dinner at Highseas

7:00pm Workshop for genetic, phenotypic, and imaging databases – Janan Eppig  
Highseas lecture room

***Tuesday, September 28***

7:00am Breakfast at Highseas

7:50am Shuttles to lab

8:00am-5pm Modules; continuing in three teams, members will continue

- Mouse biology and germline genetics (Module 1)
- Cellular and tissue biology (Module 2)
- Molecular genetics (Module 3)

12:00-1:00pm Lunch, all together for “show and tell”, GRB 3225 conference room

- Transfection of single cell preparations – Jeff Franklin
- Markers for stem cells, progenitors – Robert Whitehead, Jeff Franklin, Xiaodi Chen, Rich Halberg
- Proliferative and apoptotic indices – Xiaodi Chen
- Cell migration – David Threadgill, Erica Rinella

6:00pm Dinner at Highseas

7:00pm Cluster analysis of array data in the mouse and the human – Bruce Aronow and Tim Yeatman:

***Wednesday, September 29***

7:00am Breakfast at Highseas

7:50am Shuttles to lab

8:00am-5pm Modules; the three teams of students will continue:

- Mouse biology and germline genetics (Module 1)
- Cellular and tissue biology (Module 2)
- Molecular genetics (Module 3)

12:00-1:00pm Lunch, plenary session, GRB 3225 conference room

- Preparation of mosaics and embryo fusion chimeras – Rich Halberg and Kevin Haigis
- The elements of pathway and network analysis and the emergent resources in mouse biology and genetics – Bill Dove and David Threadgill

4:30-5:00pm Shuttles to Highseas

6:00pm Lobster Dinner at Highseas jointly with the Molecular Histopathology Group

***Thursday, September 30***

7:00am Breakfast at Highseas

7:50am Shuttles to lab



- 8:00am Participants will be able to fill in gaps of their command over the Experimental Methods by repeating certain steps that may have failed in the first attempt.
- 10:00am Show-and-tells on immunohistochemistry for molecular markers in the Apc/Wnt domain – Inke Nathke and Maaïke van de Born
- 11:50am Shuttles to Highseas
- 12:00 noon Lunch with the Molecular Histopathology Consensus Group, Highseas
- 1:00pm Kay Washington, Bob Coffey, and Bill Dove will meet with the participants to discuss the summary made by the Molecular Histopathology Consensus Group, Highseas lecture hall.
- Free afternoon in Acadia, with a range of individual or group activities available
- Evening: Conference on Colon Cancer in Humans and Murine Models begins

## Module 1. Mouse biology and germline genetics

### Isolation and analysis of the intestinal tract and its lesions

Dove lab (Sample images are available at <http://mcardle.oncology.wisc.edu/dove/Protocols.htm>)

#### Dissecting

1. Sacrifice mouse by CO<sub>2</sub> asphyxiation.
2. Wet fur in the abdominal region with 70% ethanol.
3. Make an incision in the skin that transverse the abdomen.
4. Pull skin so that the body wall is exposed from forelimbs to hind limbs.
5. Make an incision in the body wall that transverses the abdomen.
6. Remove the intestinal tract.
  - a. Cut the colon as near to the rectum as possible.
  - b. Grasp the colon with the dissection forceps.
  - c. Pull gently to release colon.
  - d. Grasp mesentery that is attached to the small intestine near the cecum.
  - e. Continue to pull to begin stripping off the mesentery.
  - f. Grasp small intestine with dissection forceps.
  - g. Continue to pull to release the small intestine.
  - h. Repeat in a hand over hand fashion up to the stomach.
  - i. Cut the stomach at the junction with the small intestine.
  - j. Place intestine in PBS.
7. Cut small intestine at the junction with the cecum.
8. Flush small intestine with PBS.
  - a. Attach a reusable feeding needle to a 10 ml syringe.
  - b. Draw up PBS.
  - c. Insert needle in the small intestine.
  - d. Wash out contents of the intestinal lumen by injecting PBS.
9. Cut small intestine into four equal segments. Lay four segments on a paper towel and trim off any remaining mesentery.
10. Rinse segments in PBS and lay them onto a piece of bibulous paper.
11. Cut longitudinally the entire length of each segment using microdissecting scissors with probed points. Avoid cutting tumors in half if possible.
12. Dip finger in PBS and run the length of each segment so that the intestine is flattened out. Villi and tumors should be exposed.
13. Cut colon at the junction with the cecum.
14. Wash the colon in PBS and place it on bibulous paper.
15. Cut longitudinally the entire length of the colon.
16. Remove fecal pellets with dissection forceps.
17. Dip finger in PBS and run the length of the colon to flatten it out.

18. Wash segments and colon with 70% ethanol while running finger up and down the length of each segment and colon.
19. Cut bibulous paper into three strips: one for the colon and two for the small intestine.
20. Fold each strip in half or thirds so that the sample will fit into a scintillation vial containing 10% formalin.
21. Fix tissue overnight.
22. Replace 10% formalin with 70% ethanol.
23. Tissue can be stored in this manner for years. Long-term storage requires sealing the cap of the scintillation vial with parafilm.

### Notes

1. The intestinal tract can tear quite easily, especially in mice that have several tumors.
2. Tumors in the colon can be dislodged very easily as they are attached to the mucosa by a stem-like structure.

### Supplies

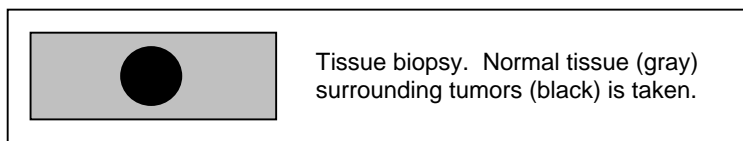
<i>Item</i>	<i>Amount</i>	<i>Source</i>	<i>Catalog No.</i>	<i>Price</i>
Forceps, Microdissecting	2	Roboz	RS-5130	\$37.20
Scissors, Veterinary	1	Roboz	RS-6940	\$101.50
Scissors, Microdissecting	1	Roboz	RS-5910	\$59.50
Scissors, Microdissecting with probed points	1	Roboz	RS-5990	\$78.80
Needle, Reusable feeding (20 gauge)	1	Roboz	FN-7921	\$34.50
Bibulous paper, (15cm x 10cm), pk of 12		Fisher	11998	\$44.37
Syringe, 10ml, pk of 100		Fisher	14-823-2A	\$20.17
Petri dish, (100mm x 15mm), case of 500		Fisher	08-757-12	\$115.06
Scintillation vials, case of 500		Fisher	03-337-4	\$167.25
Wash Bottle, pk of 4		Fisher	03-409-10D	\$16.51
Paper toweling				

### Scoring Intestinal Adenomas

1. Remove intestinal tract from the scintillation vial.
2. Remove each segment of the small intestine and colon from the bibulous paper and place them in a large petri dish containing 70% ethanol.
3. Trim off any mesentery that may interfere with scoring intestinal tumors.
4. Score the number of intestinal tumors in each segment of the small intestine and the colon under 10x magnification. Difficulties include confusing either the ampulla of Vater, cystic crypts, or lymph nodes as tumors and discriminating between tumors that are tightly clustered.

## Isolating and Embedding Intestinal Adenomas

1. Individual tumors can be isolated for histological examination using microdissecting scissors.

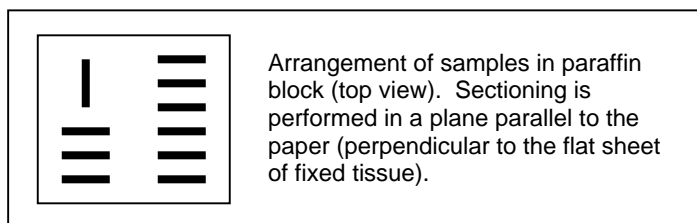


2. The biopsy is embedded in paraffin.

<u>Station</u>	<u>Time</u>	<u>Temp</u>	<u>Vacuum/Pressure</u>	<u>Solution</u>
1	DELAY	OFF	ON/ON	70% EtOH
2	OFF	OFF	OFF	
3	1:00	OFF	ON/ON	70% EtOH
4	1:00	OFF	ON/ON	80% EtOH
5	1:00	OFF	ON/ON	95% EtOH
6	1:00	OFF	ON/ON	100% EtOH
7	1:00	OFF	ON/ON	100% EtOH
8	:30	OFF	ON/ON	Xylenes
9	:30	OFF	ON/ON	Xylenes
10	1:00	OFF	ON/ON	Xylenes
11	:30	58	ON/ON	Paraffin
12	:30	58	ON/ON	Paraffin
13	OFF	58	OFF	Paraffin
14	1:00	58	ON/ON	Paraffin

Embedding protocol

3. Manipulations may vary with the experiment. We have found it convenient to embed several tumors from an individual mouse in one block. A segment containing both normal and tumor tissue is fixed flat on bibulous paper. It is then peeled off and embedded in paraffin. Different samples are arranged in a pattern to facilitate identification. The tissue is sectioned along the crypt-villus axis of the normal epithelium by cutting perpendicular to the flat fixed tissue.



### Supplies

<i>Item</i>	<i>Amount</i>	<i>Source</i>	<i>Catalog No.</i>	<i>Price</i>
Tweezers, Dumont #5	2	Roboz	RS-5045	\$28.60
Scissors, Microdissecting	1	Roboz	RS-5620	\$285.40
15 mm x 150 mm petri dish	Case of 100	Fisher	08-757-14	\$116.82

### Staining sections with hematoxylin and eosin

1. Deparaffinize sections in three changes of xylene for two minutes each.
2. 30 dips in 2 changes of absolute alcohol
3. 15 dips, then 30 sec in 80% ethanol.
4. 15 dips, then 30 sec in 70% ethanol.
5. Distilled H<sub>2</sub>O for 1 minute. Repeat four times.
6. Stain in Shandon Instant Hematoxylin for 30 sec.
7. Wash in tap H<sub>2</sub>O for 3 min.
8. 3 dips in 1% alcoholic HCl.
9. Rinse in tap H<sub>2</sub>O for 1 min.
10. Wash sections in 0.4% sodium acetate for 3 min.
11. 10 dips in H<sub>2</sub>O. Repeat three times.
12. Wash sections in 50% ethanol for 30 sec.
13. Stain in eosin-multichrome stain for 30 sec.
14. 30 dips in absolute ethanol. Repeat three times.
15. Coverslip.

### Solutions

#### *Shandon Instant Hematoxylin*

Mix parts A and B dry concentrate in 1 L of distilled H<sub>2</sub>O.  
Wait at least 12 hours and filter.

#### *1% Alcoholic HCl*

10 ml      Concentrated HCl  
300 ml     Distilled H<sub>2</sub>O  
700 ml     Absolute ethanol

Add HCl to distilled H<sub>2</sub>O, the absolute ethanol.

#### *0.4% Sodium Acetate*

1 g          Sodium acetate  
250 ml      Distilled H<sub>2</sub>O

Mix well and pH to 7.25. Make fresh.

#### *Eosin-Multichrome Stain*

2.5 g        Eosin Y  
0.25 g       Phloxine B  
500 ml      Distilled H<sub>2</sub>O  
500 ml      Absolute ethanol  
2 ml         Glacial acetic acid

Dissolve eosin and phloxine B in distilled H<sub>2</sub>O.  
Add ethanol, then glacial acetic acid. pH must be 5.

## Detecting Specific Members of the GI Flora

Deloris Alexander, Threadgill lab

### Supplies/Reagents

Fecal pellets

1.5ml Eppendorf tubes

Lysis buffer

QIAamp DNA Stool kit (Qiagen)

TE (10mM Tris, 1mM EDTA, pH 8)

10X AmpliTaq Gold buffer

25mM MgCl<sub>2</sub>

10mM dNTPs

0.2ul/ul forward and reverse primers specific for bacterial species of interest

UNG (Amp Erase)

AmpliTaq Gold

PCR machine

Agarose

TBE

Gel box

Ethidium bromide

Brucella agar plates

Incubator at 37°C

### Procedure

#### *DNA Preparation*

1. Collect 1-3 fresh fecal pellets from mice and place into 1.5ml Eppendorf tubes.
2. Add 500 ul lysis buffer and disperse by repeatedly pipeting and vortexing.
3. Isolate DNA with QIAamp DNA Stool kit according to manufacturer's procedure.
4. Resuspend DNA in TE and store at -20°C.

#### *PCR*

1. Set up PCR:
  - 3.0 ul 10X AmpliTaq Gold buffer
  - 6.0 ul 25mM MgCl<sub>2</sub>
  - 3.6 ul 10mM dNTPs
  - 1.0 ul 0.2ug/ul forward and reverse primer
  - 0.2 ul UNG
  - 0.15ul AmpliTaq Gold
  - 1.0 ul DNA suspension
  - 15.0 ul H<sub>2</sub>O
2. Run PCR reaction:
  - 50°C, 2min
  - 94°C, 10min
  - 94°C, 15sec
  - 60°C, 1min 40 cycles

*Detection*

1. Run 10ul of PCR reaction on 1% agarose gel.
2. Visualize with EtBr staining.

*Bacteria Diversity Detection*

1. Streak suspended fecal pellets onto LB plates and set a 37°C overnight.
2. After 24 and 48hrs, visual colony diversity.

References in appendix

1. [Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, and Fox JG. 1999. Phylogeny of the defined murine microbiota: altered Schaedler flora. \*Appl Environ Microbiol\* 65:3287-3292.](#)
2. [Salzman NH, de Jong H, Paterson Y, Harmsen HJ, Welling GW, and Bos NA. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. \*Microbiology\* 148:3651-360.](#)
3. [Sarma-Rupavtarm RB, Ge Z, Schauer DB, Fox JG, and Polz MF. 2004. Spatial distribution and stability of the eight microbial species of the altered Schaedler flora in the mouse gastrointestinal tract. \*Appl Environ Microbiol\* 70:2791-2800.](#)

## **Syngeneic transplants to generate metastatic lesions**

Erica Rinella, Threadgill lab

### Supplies

Cells

Sterile 1xPBS

15ml conical tubes

Ice

Oxygen

Isoflurane

Isoflurane vaporizer with flowmeter, non-rebreathing tubing, nose cone

Ophthalmic ointment

Clippers or depilatory (i.e., Nair)

Sterile gloves

Sterile drapes

70% EtOH

Betadine

Sterile swabs and/or gauze

Heating pads

Fine scissors (1) [FST 14060-09]

2.5-4mm forceps (1) [FST 11000-12]

Absorbable sutures (Ethicon Chromic Gut)

0.8mm forceps: 1 curved, 1 straight [FST 11052-10, 11050-10]

Skin stapler, clips, and clip remover [FST or Roboz]

25 gauge needles with 1 ml syringes

27 gauge needles with 1 ml syringes

0.9% saline (sodium chloride solution)

Mouse restrainer (for tail vein injections)

### Procedure

#### *Cell Culture*

1. Trypsinize cells, resuspend in media, and count cells using hemacytometer.
2. Dilute cells in sterile 1xPBS to  $1 \times 10^6$  cells/ml in a 15 ml conical tube and store on ice.

#### *Anesthetizing/prepping the mouse*

1. Turn on oxygen tank; turn on vaporizer oxygen flowmeter to 0.4-0.6. Turn on vaporizer to 2.5% isoflurane.
2. Place mouse in chamber until anesthetized (stops moving; slow, steady breathing).
3. Turn isoflurane down to a maintenance level of about 1.5% (depending on strain of mouse). This level has worked for Balb/c and C57BL/6 mice.
4. Remove mouse from chamber and place on prep area with face in nose cone.
5. Apply ophthalmic lubricant to eyes with sterile swab.



6. Use clippers or depilatory to remove hair from abdomen.
7. Clean shaved area, alternating 70% ethanol and betadine 3 times, using sterile gauze or swabs. Start at center (where incision will be made) and wipe outwards.
8. With forceps, pinch paws and/or abdomen to verify that mouse is completely anesthetized before beginning surgery.

*Transplant of syngeneic cell line*

1. Surgery should be performed on clean surface, wearing sterile gloves. Mouse should be on heating pad or under lamp providing sufficient heat so body temperature does not drop while under anesthesia.
2. Make ~1 cm incision in left abdomen just above left hind leg with fine scissors or scalpel.
3. Pinch inner layer and use fine scissors to make ~1cm incision into peritoneal cavity and expose cecum.
4. Holding the end of the cecum with 2.5-4mm forceps, use a 25 gauge needle to inject 100 $\mu$ l of 1xPBS containing 1x10<sup>5</sup> cells into the seromuscular layer of the cecum. Cecum will inflate if injection is successful.
5. Using a sterile swab gently wipe the cecum and surrounding area where cells may have spilled.
6. With blunt forceps gently return cecum to peritoneal cavity.
7. Holding inner and outer layers together, close incisions with skin stapler and 6.5-7mm clips.
8. Inject 1 ml warm 0.9% saline (~37°C) subcutaneously in 2 different areas of the back.
9. Let mouse recover on heating pad until becomes conscious before returning to a clean cage.
10. Monitor mouse for 48 hours.
11. Remove staples 1 week later.

*Tail vein injection of substrate for luciferase detection*

1. Put mouse in restrainer face-down. You can mark the top vein on the tail with a sharpie to keep track of orientation.
2. Do not inject the veins at 12 o'clock (top) or 6 o'clock (bottom). Can inject veins on the sides (3 and 9 o'clock).
3. Warm tail with warm water, heat lamp, or heating pad in order to make the veins more prominent.
4. Starting at the end of the tail furthest from the mouse use a 27 gauge needle to do the injection (avoid air bubbles).
5. Hold the tail straight, slide the needle into vein and inject. If the needle is in the vein the solution will go in easily and the blueness of the vein will disappear. If the needle is subcutaneous the solution will not go in easily and a clear bubble will form in the tail.
6. Once the injection is made hold your thumb over the needle as you remove the needle from the tail in order to prevent the solution from coming back out.

References in appendix

1. [Corbett TH, Griswold DP, Roberts BJ, Peckham JC, and Schabel FM. 1975. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Research 35:2434-2439.](#)
2. [Brattain MG, Strobel-Stevens J, Fine D, Webb M, and Sarrif AM. 1980. Establishment of mouse colonic carcinoma cell lines with different metastatic properties. Cancer Research 40:2142-2148.](#)
3. [Ray P, De A, Min J-J, Tsien RY, and Gambhir SS. 2004. Imaging tri-fusion multimodality reporter gene expression in living subjects. Cancer Research 64:1323-1330.](#)

## ***In vivo* imaging with Xenogen IVIS system**

Erica Rinella, Threadgill Lab

### Supplies

#### [IVIS imaging system](#)

Luciferin

### Procedure

#### *IVIS Anesthesia System*

1. Turn on the **Evacuation Pump** on the front panel of the anesthesia unit.
2. Turn on Oxygen (tank or supply source—should be at about 55 psig pressure).
3. Turn on **Gas Flow** on right side of imaging chamber.
4. Turn on Oxygen gas flow (**green key**) to anesthesia system.
5. Make sure vaporizer is set to the 0% position.
6. Turn on toggle valve (**IVIS Flow On/Off**) and set to 0.25 LPM/5 mice.
7. Turn on toggle valve (**Chamber On/Off**) and set to 1.5 L/min.
8. Confirm flow rate and turn off the two toggle valves.
9. Set vaporizer to 2.5%.
10. Put mice into Induction Chamber.
11. Turn on **Chamber** toggle valve to anesthetize mice (usually about 2-5 min).
12. Turn on **IVIS** toggle valve.
13. Turn off **Chamber** toggle.
14. Transport animal to restrainer for tail vein injection of luciferase substrate.

#### *Acquiring Luciferase Image*

(Can do steps 1-7 before injecting mice with the substrate)

1. Launch **Living Image** software.
2. Select User ID.
3. **Initialize** system.
4. Wait until temperature indicator is green.
5. Adjust settings according to preference. For **Luminescent** detection the following is recommended for initial image: Exposure = 1 min; Binning = medium; f/stop = 1.
6. Adjust FOV (field of view) according to the number of mice (5 is the max). **D** is the largest FOV; **A** is the smallest.
7. Select **Overlay**.
8. Move animal to nose cone, close door to imaging chamber, and **Acquire** image.
9. A black and white image will appear within a few seconds. If the FOV is not satisfactory, select **Stop** or open IVIS door to stop exposure, readjust, and **Acquire**.
10. Choose **Living Image** menu and **Save Living Image Data** to save raw, analyzed, or photographic data.
11. When images are analyzed and saved, close Living Image software.

*After Acquiring Image*

1. Turn off vaporizer.
2. Turn on **Chamber On/Off** toggle valve for 5 min and then turn off.
3. Turn off oxygen source.
4. Turn on the two toggle valves until pressure is down to 0.
5. Turn off the Oxygen valve (**green key**).
6. Make sure that all toggle valves are off and turn off **Evacuation Pump**.

## **Modifier genetics, genotyping, and analysis software**

General topics in modifier analysis will be discussed and a model dataset will be analyzed using QTX. There will be an opportunity to use data collected during the workshop for perform additional modifier mapping. See reference materials in appendix:

1. [Manly KF, Cudmore RH, and Meer JM. 2001. Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 12:930-932.](#)
2. [Chmielewicz KM and Manly KF. 2002. Tutorial for QTX.   
<http://www.mapmanager.org/mmQTX.html>](#)
3. [Broman KW, Wu H, Sen S, and Churchill GA. 2003. R/qtl: QTL mapping in experimental crosses. Bioinformatics 19:889-890.](#)
4. [Threadgill DW, Hunter KW, Zou F, and Manly KF. 2004. Genetic Modifiers in “Mouse Models of Human Cancer”, Edited by EC Holland: John Wiley and Sons, New York.](#)

Additional reference on the web:

Chielewicz KM and Manly KF. 2002. QTX manual.  
<http://www.mapmanager.org/mmQTX.html>

## **Module 2. Cellular and tissue biology**

### **A clonogenic assay for identifying growth and differentiation factors for colonic crypt epithelium**

Robert H. Whitehead and Jeffrey L. Franklin

#### Introduction

Although the intestinal epithelium proliferates rapidly throughout life, very little is known about the factors that control this process. Because of the difficulty we have encountered in culturing colonic epithelial cells most of our knowledge about growth control of the colon come from *in vivo* studies. The problem with these studies is that it is impossible to be certain that you are seeing a primary effect and not some secondary effect caused by the factor you have administered.

It is well demonstrated that each crypt of the colonic mucosa contains a small proportion of stem cells from which all of the cells of the mucosa arise. In the hemopoietic system it has been possible to develop assays for the stem cells and to identify the factors that induce both proliferation and differentiation into the known mature cell phenotypes of the blood and bone marrow.

A similar assay for colonic stem cells could provide a method for identifying similar factors for this tissue. I will demonstrate a method that I have devised for isolating crypts free from stroma and for disaggregating these crypts into single cells. I will then use these single cells in a clonogenic assay based on the hemopoietic stem cell assay. We can use this assay to identify growth factors for the proliferative cells of the colonic crypt.

## Supplies

Mice (3 per student)

Dissecting instruments (forceps and small scissors – 1 set per student).

50ml centrifuge tubes

0.04% sodium hypochlorite in PBS

3mM EDTA plus 0.5mM DTT in PBS (sterile).

PBS (sterile).

Clean 500 ml beakers

0.3% pancreatin (Sigma Cat No. P1500). The solution is allowed to dissolve for 2-3 hours then filtered and aliquoted in 25ml volumes in 50ml centrifuge tubes. Store at -20°C until used.

1% agar in H<sub>2</sub>O – aliquoted in 50 ml volumes (sufficient for 1 experiment) and autoclaved.

0.7% agarose type VII (Sigma Cat. No. A4018) in H<sub>2</sub>O- aliquoted in 50 ml volumes and autoclaved.

RPMI1640 plus 5% FCS

x2 RPMI1640 plus 2 µg/ml insulin and 10% FCS

6 well tissue culture plates or 35 mm tissue culture dishes.

15ml centrifuge tubes.

21 gauge needles

23 gauge needles.

10ml syringes

Sterile 5 ml screw capped tubes.

Plugged, sterile Pasteur pipettes.

Bench top centrifuge

Hemocytometer

Waterbath

Microscope

### **Isolation of colonic crypts and preparation of a single cell suspension**

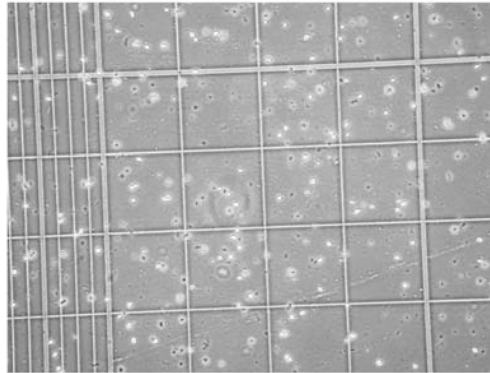
1. The mice (normally 3 per assay) will be euthanised using standard procedures.
2. The abdomen will be swabbed with alcohol and then opened and the colon removed.
3. The colons will be opened along their length and washed 3 times in a beaker containing PBS.
4. The colons are rapidly transported to the laboratory in  $\text{Ca}^{++}$  free -PBS and transferred to 25 ml of 0.04% sodium hypochlorite in PBS (3 colons can be incubated in this volume) in 50 ml screw capped tubes and incubated at room temperature for 15 minutes.
5. The sodium hypochlorite is discarded and the colons are washed twice in PBS.
6. **Note:** After this step, the colonic tissue is considered to be sterile and all subsequent procedures are carried out with sterilized reagents.
7. The PBS is discarded and replaced with 25 ml of sterile 3mM EDTA plus 0.5mM DTT in PBS and incubated for 90 minutes at room temperature.
8. The EDTA/DTT solution is discarded and 20ml of sterile PBS is added. The screw cap is tightened and the tube is shaken VIGOROUSLY for 15-20 seconds. The PBS containing crypts is transferred to 15 ml centrifuge tubes and the process repeated by adding 15 ml of PBS to the tubes and repeating the shaking process.
9. The tubes containing the crypt suspension are centrifuged gently (at 400rpm) for 5 minutes.
10. The PBS is removed and the crypts are resuspended gently in the pancreatin solution. The tube is shaken gently to disperse the crypts and incubated at room temperature for 90 minutes.
11. The tube is shaken gently every 10 minutes. If the crypts are clumping they can be dispersed by slightly more vigorous shaking or very gentle pipetting.
12. From 60 minutes the disaggregation of the crypts can be monitored by collecting a drop of crypt suspension and examining it under a microscope. The crypts should have broken up to single cells and very small crypt fragments.
13. After 90 minutes (or before if a good disaggregation is obtained) the crypt suspension is diluted with an equal volume of PBS and transferred to 15ml centrifuge tubes and centrifuged at 1000rpm for 5 minutes.
14. The supernate is discarded and the cell pellet is resuspended in EDTA/DTT solution and centrifuged at 1000rpm for 5 minutes.
15. The pellets are pooled in 5 ml of RPMI1640 plus 5% FCS and resuspended by gentle pipetting. The suspension is transferred to a 5ml tube.
16. The cell suspension is examined under the microscope. If cell clumps are present the suspension is passed gently through a 21 gauge needle attached to a 10ml syringe. This is repeated 3 times **very gently** followed by three times through a 23 gauge needle. **NOTE:** - The cells are very fragile at this stage and goblet cells will readily release their mucin trapping all the cells in an unusable clump.



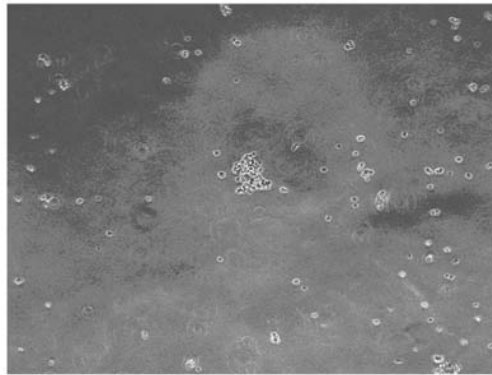
17. If cell clumps are still present, they can be removed by passing the cell suspension through a filter made by gently pushing the cotton wool plug in a Pasteur pipette down into the barrel of the pipette. The pipette is placed upright in a 15ml tube and the cell suspension is added slowly and allowed to drain through the cotton wool plug. This procedure results in the loss of approximately 50% of the cells however.
18. The cell suspension is counted using a hemocytometer and the cell count adjusted to  $5.0 \times 10^5$  viable cells per ml with RPMI 1640 plus 5% FCS.

### The clonogenic assay

1. While the cells are incubating in pancreatin the agar and agarose are prepared and the growth factors being tested are thawed.
2. The agar and agarose are melted (normally in a microwave oven –taking care to loosen the bottle top and handling the bottle containing the molten agar carefully) and cooled to 41°C in a waterbath. 2x RPMI1640 containing 2ug/ml insulin and 10% FCS is warmed to 41°C in the waterbath.
3. An underlay is prepared by mixing the agar with an equal volume of 2x RPMI1640 and dispensing 2ml into each well or plate. Allow the agar to set.
4. Sufficient 10 ml tubes for all of the factors being tested plus a control are labeled and a volume of factor to give the required final concentration in the agarose/medium (normally 5ml) is added to the appropriate tube.
5. The agarose is mixed with an equal volume of 2x RPMI1640 plus FCS and insulin and 5ml is aliquoted into each tube. The tubes are kept at 37°C to prevent gelling.
6. 0.5ml of cell suspension is added to each tube and the tubes are mixed well.
7. The mixture in the tubes is aliquoted into labeled wells in 2 ml volumes.
8. The plates are cooled to below 24°C to allow the agarose to gel.
9. The plates are placed in a plastic container containing a small container of H<sub>2</sub>O and sealed with a lid.
10. \*Some studies in other organ systems have suggested that better cloning efficiencies are obtained if the container is gassed with a low O<sub>2</sub> gas mixture. Normally 5% O<sub>2</sub> is used in a mixture with 5% CO<sub>2</sub> and 90% N<sub>2</sub>).
11. The containers are incubated at 37°C for 3-4 weeks.
12. The plates are examined using either a dissecting microscope or an inverted microscope and searched for colonies.
13. Aggregates of more than 30 cells are scored as colonies. Aggregates of between 15 and 30 cells are scored as clusters.
14. If required, colonies can be removed from the agarose with a fine-tip pipette and transferred to fixative for histology or solution D for RNA studies.
15. **Note:** This is an evolving technique. The method described here is not necessarily the optimal method. Further experimentation may make improvements in crypt disaggregation, culture methods and medium and methods of assessing colony formation. Similarly better methods for assessing colony phenotype are needed.



Single Cell Preparation



Colony in agarose

## References

1. Whitehead, R.H., Brown, A. and Bhathal, P. (1987) A method for the isolation and culture of human colonic crypts in collagen gels. *In Vitro* 23:436-442.
2. Whitehead, R.H. and Van Eeden, P.E. (1991) A method for the prolonged culture of colonic epithelial cells. *J. Tissue Culture Methods*, 13: 103-106.
3. Whitehead, R.H., Van Eeden, P.E., Noble, M.D., Ataliotis, P. and Jat, P.S. (1993) The establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *PNAS*, 90, 587-591.
4. Whitehead, R.H. and Joseph, J.L. (1994) Derivation of conditionally immortalised cell lines containing the Min mutation from the normal colonic mucosa and other tissues of an F1 mouse (Immortomouse/Min). *Epithelial Cell Biology* 3, 119-125.
5. [Whitehead, R.H., Demmler, K., Rockman, S.P. and Watson, N.K. \(1999\) Clonogenic growth of epithelial cells from normal colonic mucosa from both mouse and human. \*Gastroenterology\*, 117, 858-865.](#)
6. [Fujimoto, K., Beauchamp R.D., Whitehead, R.H. Identification and isolation of candidate human colonic stem cells based on cell surface integrin expression. \*Gastroenterology\*, 123, 1941-1948, 2002.](#)

### **Fractionation of the Cell Suspension on a Density Gradient**

Demonstration – Jeff Franklin

The single cell suspension is gently layered over an Optiprep step gradient of 10% (5ml) over 20% (4ml) at a cell concentration of  $2 \times 10^6$  per ml and centrifuged at 840 g for 30 minutes. The separate cell layers are carefully removed using a pipette and transferred to 15 ml centrifuge tubes containing 10 ml of medium. The tubes are centrifuged at 1000 rpm for 5 minutes. The supernate is discarded and the cells resuspended in 2ml of medium, counted and plated in the standard clonogenic assay.

### **Cell Sorting with Antibodies or Dynabeads**

Discussion – Bob Whitehead

Once a single cell suspension is obtained, the cells can be fixed in 4% paraformaldehyde (or other fixative) and tested for expression of various antigens using appropriate antibodies. The cells can either be stained in suspension or cytopsin preparations can be prepared. Once fixed, standard immunohistochemical methods can be used for staining. If expression of membrane epitopes is the focus of the experiment, the cells can be stained as a viable cell suspension. In this case, the cells must be kept at 4°C throughout the staining procedure to avoid antigen capping and internalization or membrane shedding. These cells can then be sorted using standard fluorescence activated cell sorting (FACS) techniques. If the sorting is done using sterile techniques then the sorted cells can be assayed in the clonogenic assay.

Alternatively subpopulations of cells expressing a particular epitope can be isolated using Dynabeads (DYNAL Corp). These are small magnetized beads that can be conjugated with an antibody and then mixed with the single cell suspension. After incubation at 4°C, the tube containing the cell/bead mixture is placed in a magnet and all the non-reacting cells can be removed. The tube is removed from the magnet and the cells are washed with cold PBS. The cells expressing the epitope (and the negative cells) can then be assayed as 2 subpopulations in the clonogenic assay.

Alternatively the subpopulations of cells could be used as a source of RNA for microarray studies or other studies looking for unique gene expression patterns.

### Module 3. Molecular Genetics

XC, WFD, RBH Core Staff

JBM The Jackson Laboratory Coordinator

Inke Nathke and Maaïke van den Born – Thursday Show and Tell re IHC

In situ hybridization (ISH) and immunohistochemistry (IHC) require overnight incubations followed by development (4 hr). On Sunday afternoon, Workshop Staff will set up the overnight incubations for Monday's development of ISH and IHC by the Trojan team. The Trojan team will in turn set up overnight incubations for ISH and IHC on Monday afternoon for the Roman team to develop on Tuesday. The Roman team will then set up ISH and IHC on Tuesday afternoon for the Greek team to develop on Wednesday. Finally, the Greek team will set up ISH and IHC on Wednesday afternoon for any course member to develop on Thursday as needed. One half of a team will perform ISH and the other half will perform IHC. The results of these two modes of analysis will be compared.

*Friday 9/24:*

Preparation of DEPC-treated buffers for ISH by JBM and his staff.

After overnight DEPC treatment, buffers need to be autoclaved.

Protocol ISH – Core

*Sunday 9/26:*

Ca 2 PM RH, WFD, and XC arrive at The Jackson Laboratory

Make other buffers for ISH, IHC

Module 3 ISH protocol, XC

Module 3 IHC protocol, RBH

Setup Day 1 incubations for **Team T**

Module 3 ISH protocol, XC

Module 3 IHC protocol, RBH

*Monday 9/27:*

8:30 **Team T1:** Day 2 ISH development

Module 3 ISH protocol, XC

**Team T2:** Day 2 IHC development

Module 3 IHC protocol, RBH

Lunch will be taken by course members ad lib, as time permits.

1:30 **Team T1:** Setup Day 1 incubations of ISH for **Team R1**

Module 3 ISH protocol, XC

**Team T2:** Setup Day 1 incubations of IHC for **Team R2**

Module 3 IHC protocol, RBH

During incubation intervals, Team T1 will discuss the outcome of their ISHs and Team T2 will discuss the outcome of their IHCs.

Ca 4:30 **Team T:** Wrapup discussion of ISH versus IHC in the Conference Room with XC, RBH, and WFD

*Tuesday 9/28 and Wednesday 9/29:*

8:00 – 12:00 and 1:00 – 5:00

On Tuesday, **Team R** will follow the pattern outlined above.  
On Wednesday, **Team G** will follow that pattern.

*Tuesday 9/28:*

12:00 – 1:00 Plenary lunch in Conference Room:  
Show-and-Tell of protocols in cell biology  
Proliferative and apoptotic indices – XC  
DNA transfection of cultured colonocytes - JF

*Wednesday 9/29:*

12:00 – 1:00 Show-and-Tell of protocols and concepts in functional analysis  
with the biology and genetics of the laboratory mouse  
Chimeras – RBH  
Pathway and network analysis – WFD, DT

*Thursday 9/30:*

8:00 – 10:00 (as needed) Course members can repeat Day 2 ISH and/or IHC  
development, using samples prepared on 9/29 by **Team G**.  
10:00 – 11:50 Conference Room: Show-and-Tell for all course members  
IHC for APC/Apc by Inke Nathke  
IHC for Wnt-pathway molecules by Maaïke van den Born

## **ISH: Non-radioactive *in situ* hybridization of paraffin embedded tissues**

Updated by Xiaodi Chen and Bill Ehrhardt 2/13/03; short version 9/16/04.

Modified from Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J. & Kern, S. E. *Cancer Res.* 62:5351-5357 (2002)

*Part A: Digoxigenin-labeled riboprobes will be provided by the instructor*

*Part B: In Situ Detection*

### **Day 1**

*The following steps are carried out in 50 ml conical tubes. Each 50ml tube can hold 2 outward-facing slides. 30ml of solution is sufficient to immerse sections.*

*Prior to starting dewaxing steps, prewarm citrate buffer to 95 °C for Step #6. Also, preheat hybridization ovens to chosen hybridization temperature (see step 15).*

*All buffers and solutions used on Day 1 must be **RNase-free**.*

1. Have your paraffin embedded sections cut onto microscope slides that are polylysine coated or charged. Once cut, these sections can be stored in any clean, dry container until ready for use. (Provided by the instructor)
2. Incubate slides in xylene 2 x 5 minutes with shaking.
3. Incubate in 100% ethanol 5 minutes with shaking.
4. Incubate in 70% ethanol 5 minutes with shaking.
5. Incubate in 50% ethanol 5 minutes with shaking.
6. Rinse in dH<sub>2</sub>O twice for 5 min., then put into pre-warmed citrate buffer (0.01M, pH 6.0, fresh), incubate at 95°C for 20 min.
7. Cool down the slides to room temperature in the same buffer (approx. 5 min.).
8. Incubate 5 minutes in TBS.
9. Immerse sections in Proteinase K (see stocks) diluted in 35 ml. fresh TBS (started from 5µg/ml). Incubate for exactly 5 minutes at room temperature. (use 5-8uL proteinase K depending on new or old stock)
10. Rinse in TBS twice, then incubate in 3% hydrogen peroxide 10 minutes RT (made by diluting 1:10 commercial 30% Hydrogen Peroxide with TBS). 30ml TBS added to 5ml Hydrogen Peroxide.

11. Immerse slides into 0.1M triethenolamine solution, add acetate anhydrate to final concentration of 0.25% and immediately mix the solution well immediately. Incubate for 10 minutes at RT. Add 80ul anhydrate to 30ml TBS.
12. Place slides in TBST at RT.
13. (Can be done during the incubation of step 10 and 11) In a 1.5ml RNase-free microfuge tube add digoxigenin-labeled riboprobe to fresh hyb. solution (DAKO, cat #S3304) (final concentration 100-500 ng/ml) ensuring a dilution of at least 1/50. Vortex. Add 0.5ul probe to 30-40ul hybridization buffer per section.
14. Briefly dry the slide around the tissue with KimWipes. Put the HybriWell hybridization chamber onto the slide, firmly press over adhesive areas of hybridization chamber while making sure not to touch the tissue (normally two chambers for two tissue sections on a slide). Carefully add 30-40ul of mixed probe from the hole of one side until the liquid reaches the other hole.
15. Denature probe at 68°C in an oven for 10 minutes and place immediately in a RNase free pre-warmed wet chamber and incubate overnight in a hybridization oven at 15-25° below the hybridization temperature (HT) predetermined for the individual probe.

## **Day 2**

*We do not use RNase free buffers after step 2. Pre-warm all wash solutions the night before.*

1. Rinse excess probe from slides by incubating in 50ml tubes containing 30ml 2X SSC for at least 5 min in a 55°C water bath.
2. Rinse with 2XSSC, then incubate in 2XSSC containing RNase A (25µg/ml, add 45ul RNase to 30ml 2XSSC) at 37°C for 15 minutes.
3. Stringently wash slides with pre-warmed 1X SSC, 50% formamide DI in a 50 ml tube (cat# AB600 American Bioanalytical) twice at 55°C for 10 min each.
4. Stringently wash slides with prewarmed Stringent Wash Buffer once for 10 min at 60°C in Hybridization oven. Make this final wash solution using Dako's 50X Stringent Wash Concentrate (which also contains detergent and blocking agent) diluted 1:50 (found in DAKO GenPoint kit, cat # K0620). Add 30ml DIW to 600ul stringent wash concentrate. Remember to pre-warm before use.
6. Rinse once with TBST for 3 min at RT.
7. Quickly dry around the sections with a Kimwipe. Next, circumscribe the outside edges of the slide with a heat-resistant pap pen (e.g. RPI's Super HT cat #195505). Before the sections dry, add 40ul of blocking buffer (see stocks). Incubate for 15 min at RT.



*Before using blocking buffer, be sure to add 1/20 dilution of rabbit immunoglobulin Fraction (DAKO cat #X0903). For 8 slides make 680ml total adding 646 $\mu$ l buffer to 34 $\mu$ l Rabbit Immunoglobulin. Make two tubes since second incubation uses same blocking buffer with HRP-anti-DIG added. We use blocking buffer with rabbit immunoglobulin for all steps in which blocking buffer is used. After making all immunoglobulin tubes vortex briefly and spin down before use.*

8. Incubate sections with rabbit HRP-anti-DIG (Dako cat # P5104) diluted 1:30 - 1:100 in Blocking buffer for 20 min at RT. We use a hybridization chamber for all incubation steps to ensure that sections do not dry out. For 8 slides add 6.8ul anti-DIG to 680ul tube of blocking buffer.
9. Wash 3 X 5 min with TBST with agitation (I use a platform shaker set to 2-3).
10. Add one drop of ready-to-use biotinyl-tyramide (from DAKO GenPoint kit) directly to slides. Incubate in DARK 10 min at RT. (Use an inverted ice bucket to accomplish this). After this step use foil covered tubes for wash steps.
11. Wash 3 X 5 min with TBST with agitation.
12. Incubate sections with one drop secondary streptavidin for 10 minutes at RT (from DAKO GenPoint Kit).
13. Wash 3 X 5 min with TBST with agitation.
14. Develop with DAB, made from diluting DAB concentrate 1:50 in the provided dilution buffer (from DAKO GenPoint Kit). Add ~25ul to each section. Color develops quickly with DAB. Monitor color development carefully and be prepared to quickly dunk slides in a Coplin jar filled with water to stop reaction.
15. In a Coplin jar, counterstain for 1-3 dips with Mayers Hematoxylin (Dako, cat # S3309). Rinse twice with dH<sub>2</sub>O.
16. Dehydrate sections in graded alcohols, 5 min. each: 70% ethanol, then 90% ethanol, followed by 100% ethanol.
17. Place in xylene 5-10 minutes.
18. Finally, place glass coverslip on a Kimwipe, add one drop of Permount (Fisher) to coverslip, invert slide and press section onto coverslip.

## ISH Supplies

### Appliances/Area:

Platform Shaker

Water Baths- 37/ 55/ 95, deep enough for 50mL conical tubes

Box for Dark room steps, clean shoe box will work

Freezer and fridge for storage

Centrifuge for 1.5mL tubes

2 hybridization ovens

Chemical hood for slide prep. and staining

Sink area for staining

Ice bucket

### Supplies/Solutions:

4 flat head tweezers

Kim wipes (small) - 6 boxes

RNase A- Roche cat# 109 169(25mg/mL)

Xylenes (500mL)

Ethanol- 95% (1L)

Proteinase K- Roche cat# 745723-1 bottle

Hydrogen Peroxide-30% (250mL) - 1 bottle

Acetic Anhydride-Sigma cat#A6404 (200mL) - 1 bottle

Triethenolamine-Sigma cat#T1377-100 (100mL) - 1 bottle

Tween 20- Fischer cat#BP337-100(100mL) - 1 bottle

RNase free conical tubes 15ml (1 pack), 50ml (2 packs of 25)

RNase free 1.5mL eppendorf tubes (1 box)

Dako GenPoint Kit for Tyramide signal amplification- cat#K0620 (1 box needed)

Sigma Hybri-well Press-seal hybridization Chamber Kit- 20mm×.15mm/ cat# H1284 (2 packs of 100 needed)

DEPC diethyl pyrocarbonate-Sigma cat# 159220-25G- (1 bottle)

2 sets of boxes for incubation steps (clean/empty p1000 tip boxes can be used)

Pipettors (p1000, p200, p100, p10)

RNase free Pipette tips from CLP, p1000, p200, p100, p10 (1 box of each)

Formamide-cat#AB600 American Bioanalytical (1L)

Heat resistant pap pen- RPI Super HT-cat# 195505 (1 pen)

Rb immunoglobulin- DAKO cat# X0903 (1 bottle)

Rabbit HRP- anti DIG- DAKO cat#P5104 (1 bottle)

Mayers Hematoxylin- DAKO cat#S3309 (1 bottle)

High purity Casein for Blocking buffer- Roche (1 bottle)

Coverslips 24×50mm (1 box)

Surgipath Mounting Medium (or Fischer Permount) for In Vitro Diagnostics- cat# MX-004 (1 bottle)

### IHC: $\beta$ -catenin immunohistochemistry protocol for paraffin-embedded tissue

From Threadgill lab, using Zymed Histomouse kit (reagents from kit in parentheses)

Kit instructions available at <http://www.zymed.com/pdf/95-xxxx/95-9541.pdf>

1. Dewax	Xylene	2x 10 min
2. Rehydrate	EtOH %-100/95/80/70/50	2 min each
	PBS-0.5% Tween20 (1.5ml in 300ml)	15 min
	PBS	5 min
3. Peroxidase quench	MeOH	2 min
	MeOH:30% $H_2O_2$ (9:1)	20 min
	MeOH	2 min
	PBS	2x 5 min
4. Antigen retrieval	250ml 10mM citrate buffer, ph 6.0	Microwave 3 min 45 sec
	PBS	2x 3 min
5. Block	BEAT Blocking Solution A (Reagent 1A)	30 min
	BEAT Blocking Solution B (Reagent 1B)	10 min
6. Primary	Transduction Labs $\beta$ -catenin antibody C19220 1:100 in 10mM PBS, to cover tissue	1 hour, humid chamber
	PBS	3x 2 min
7. Secondary	Biotinylated Antibody Mix (Reagent 1C)	30 min
	PBS	2x 3 min
8. Enzyme conjugate	ABC Reagent (Reagent 2)	20 min
	PBS	3x 2 min
9. Stain	Roche or Zymed DAB substrate* (Reagent 3A + 3B + 3C)	Up to 15 min (check staining)
	$H_2O$	Rinse
	Hematoxylin (Reagent 4)	5 dips
	$H_2O$	Rinse
	PBS	2x 1 min (check bluing)
	$H_2O$	Rinse
10. Dehydrate	EtOH %-50,70,80,95,100	30 sec each
	100% EtOH	1 min
	Fresh xylene	2x 10 min
11. Mount	Few drops Permount (Reagent 5)	Coverslip, flat overnight

\* Kit uses AEC reagent – if AEC is used do not dehydrate and mount with aqueous media.

IHC supplies

Item	Amount	Source	Catalog No.	Price
Coplin jar	8	Fisher	08-812	\$51.09 for 6
Humidity chamber	1	Shandon Lipshaw	197	\$145
Tupperware container, microwave-safe (1 liter)	1			
Weck-cel Surgical Spears	1 box of 144	Walla Medical	S-0008680	\$29.60
Coverslips				
Mounting medium				
Super HT PAP Pen, Mini	1	Research Products International	195506/T	\$45.00
Xylene (certified ACS)	4 liters			
100% EtOH	2 liters			
95% EtOH	2 liters			
90% EtOH	2 liters			
80% EtOH	2 liters			
70% EtOH	2 liters			
50% EtOH	2 liters			
Citric acid (anhydrous certified ACS)	> 20 g			
Sodium hydroxide	> 20 g			
PBS	20 liters			
Distilled water				
Hydrogen peroxide				
Methanol, 100%	4 liters			
Tween 20	> 20 ml			
HistoMouse kit, DAB (includes ABC-Amp reagents)	1	Zymed	95-9551	\$235
Monoclonal Anti Mouse B-Catenin	1	BD Biosciences	610153	\$195
Refrigerator space to hold humidity chamber overnight at 4° C.				
Microwave				

## **Plenary sessions**

### ***Monday lunch plenary session:***

#### **Realtime PCR assays of RNA levels**

Demonstration – KH

### ***Monday evening plenary session:***

#### **Public databases for genetic mapping, imaging**

Workshop – JE

MTB: Web-Based Resources for Basic Cancer Research

[http://tumor.informatics.The Jackson Laboratory.org/cancer\\_links.html](http://tumor.informatics.The Jackson Laboratory.org/cancer_links.html)

Animal Models: Distributors and Animal Production Services

Animal Models: Phenotypes for Genetically Defined and Genetically engineered Animals

Cancer Genetics and Genomics

Pathology & Images

Reagents, Services, Laboratory Protocols

Toxicology

### ***Tuesday lunch plenary session:***

#### **Transfection of single cell preparations**

Discussion - JF

The single cell suspension can also be used in transfection studies. Genes of interest can be introduced into the cells either using viral vectors or electroporation. Other techniques may also work but have not been tried. The transfected cells can be assayed in the clonogenic assay.

#### **Markers for stem cells, progenitors**

Demonstration – RW, JF, XC, RH

#### **Proliferative and apoptotic indices**

Demonstration – XC

#### **Cell migration assays**

Demonstration – DT, ER

### ***Tuesday evening plenary session:***

#### **Cluster analysis of array data in the mouse and the human**

Workshop – BA, Tim Yeatman

*Wednesday lunch plenary session:*

**Preparation of mosaics and embryo fusion chimeras**

Demonstration – RH, KH

**The elements of pathway and network analysis and the emergent resources in mouse biology and genetics**

Discussion – WD

Biological processes occur either by the transformation of material (e.g. in metabolism) or by the transfer of “information” (which often, if not always, involves material transfer as in the phosphorylation of substrate proteins during “signal transduction”). In the simplest case, such a process can be represented as a linear pathway from state A to B to C by processes alpha-beta and beta-gamma, respectively. But life is not that simple.

One source of complexity beyond the simple linear dependent pathway is that of parallel pathways, where the endstage Z can be reached in more than one way. Indeed, it has long been appreciated that metazoan development often involves “double assurance”.

Formally speaking, parallel metabolic pathways create one class of the general category of genetic redundancy. A number of other scenarios of redundancy are known, including regulatory compensation through homeostatic negative feedback (see below). In cases of formal genetic redundancy, the phenotype of a single loss-of-function mutation is masked, detectable only as a covert “internal molecular phenotype.” An overt phenotype of a redundant system often requires the construction of a multiply mutated genome.

Reference Hartwell’s SCIENCE article on redundancy  
and “synthetic lethal” genetic screens.

Another source of complexity beyond the simple linear dependent pathway is that of mutual dependence, where processes must occur together to generate the biological outcome of interest. An example illustrating both redundancy and mutual dependence is that of vision in humans. One can carry out most vision-dependent functions with either the right or left eye (redundancy). But stereoscopic vision depends on sight in both eyes (mutual dependence).

We shall discuss the available genetic tests for dependent pathways, for redundancy, and for mutual dependence in the context of the experimental biology of cancer. Genetic resources in the laboratory mouse are currently emerging to create a rich vista for the intrepid experimentalist (Three Nature Genetics commentaries attached as PDFs – Austin et al 2004; Auwerx et al 2004; Skarnes et al 2004).

A third source of complexity beyond the simple linear dependent pathway is that of feedback. A regulatory interaction, involving “information transfer”, can be either positive or negative in sign, reflecting whether state A promotes or inhibits its target process. A feedback loop is composed of a chain of regulatory interactions emerging from state A that ultimately affects the activity of state A. A feedback loop has negative

parity if it contains an odd number (1, 3, 5, etc) of negative regulatory interactions. By contrast, a feedback loop has positive parity if it contains an even number (0, 2, 4, etc) of negative regulatory interactions. Rene Thomas, a geneticist/mathematician from Brussels, has now made formal demonstrations of the properties of these two classes of feedback loop (Thomas 1998). An internal reporter of the activity of a particular gene of interest is crucial for detecting a feedback circuit involving that gene and operating in the temporal and spatial domain of interest for a cancer process of interest.

An illustrative example of feedback is evident in the recent report of Kawasaki and Taira (2004). **Question 1:** Draw the logical circuit diagram that summarizes the observations in this report.

Globally, we see that any one biological process is complex by involving one or more multistep pathways, the potential for redundancy among them, and feedback loops. Feedback loops of negative parity buffer changes in the process, while those of positive parity sharpen the decisions between alternative biological states. How can the experimentalist tease apart such complexity? How can the biomedical investigator learn what aspects of a process in cancer is susceptible to intervention?

Most strategies for experimental analysis of a biological process involve finding ways to perturb a system and then observing the response to the perturbation. The geneticist depends upon studying the consequences of introducing alternative forms of particular genes (“alleles”), either singly or in multiple combinations. In this strategy, it is important to recognize the differences between alleles with altered activity (“hypomorphs”, “hypermorphs”, and “neomorphs”) and alleles with no activity (“amorphs” and “null alleles”). It is also important to recognize the differences between studying allelic substitutions one gene at a time versus studying an ensemble of allelic substitutions. The emergent resources in mouse genetics develop different facets of these strategic considerations (Austin et al. 2004; Auwerx et al. 2004; Skarnes et al. 2004; CTC 2004).

**Question 2:** How are the interpretations of the report by Kawasaki and Taira affected if the action of the siRNA is a non-specific interference in function rather than a specific reduction in E-cadherin function? How can one test whether the siRNA effect is specific for E-cadherin?

The action of a particular gene can either be autonomous to the cell, or non-autonomous. The case of non-autonomy can be quite indirect, involving a chain of genetic activities that eventually influences distant cells in the organism. By contrast, most instances of the activity of “activated oncogenes” and “tumor suppressor genes” seem to be autonomous to the tumor cell; they can be observed in clonal cell culture. Mouse biology and genetics permits the construction of chimeras and mosaics in which cells of contrasting somatic genotype are juxtaposed in the animal. This permits tests of autonomous gene action, as illustrated by Gould and her colleagues (1997). **Question 3:** If the *Mom1* locus contains two modifying genes, rather than just one, how would the interpretation of this experiment be altered?

## References

1. [Austin C.P. et al. The Knockout Mouse Project. Nat. Genet. 36:921-924 \(2004\).](#)
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***Thursday morning plenary session:***

**IHC for APC/Apc**

Show-and-tell – IN

*In my “show and tell” session I will aim to illustrate the potential pitfalls associated with immunohistochemistry (IHC) using the p53 and Adenomatous Polyposis Coli (APC) proteins as examples. This discussion is based on data obtained in collaboration with a number of colleagues including B. Henderson and M. Brocardo (Institute for Cancer Research, and Millennium Institute at Westmead Hospital, University of Sydney, Westmead, Australia), Abby Conlin, A. J. Munro, S. Lain, D. P. Lane (Ninewells Hospital and Medical School, Dundee, Scotland), K. Haigis (Massachusetts Institute of Technology, Cambridge, USA), I. P. Newton and D. P. Lunny (Cell & Developmental Biology, University of Dundee, Dundee, Scotland).*

APC is mutated in most colonic tumours and these mutations occur early in the progression of the disease. Not surprising for a protein whose loss has an extremely highly penetrant phenotype in gut tissue, the APC protein has emerged as a multifunctional protein that is involved in a number of processes that govern the normal maintenance of gut epithelium. APC is implicated in cytoskeletal organisation so that loss of APC leads to defects in cell migration and division. In addition, APC is an integral part of a signalling pathway that affects transcription so that loss of APC also results in changes in transcriptional profiles that in turn relate to changes in differentiation and proliferation. To understand how cells distribute APC to different sites and functions, the sub-cellular localisation of APC has been investigated in detail. In addition, the ability to detect whether a tumour expresses truncated APC or arose due to other molecular changes has important implication for therapy and prognosis.

A variety of antibodies have been used to detect normal and mutated APC protein in cultured cells and in tissues. Discrepancies between results obtained with different reagents prompted us to evaluate a panel of nine different APC antibodies to determine their suitability for detecting APC protein in different sub-cellular compartments. We found that particularly for the nuclear component of APC staining, many of the available antibodies were unsuitable. This makes using them for tissue staining questionable. In addition, different fixation methods produced different patterns further illustrating how important it will be to establish standard protocols for different antibodies. With this idea in mind we have now begun to use a selected panel of APC antibodies to stain both mouse and human tissue to establish protocols that yield the most reliable results.

To further illustrate the importance of these issues, I will discuss the situation that has arisen in the case of p53. Mutations in p53 or changes in its regulation are common to many different types of cancers. Together with the importance of p53 in guarding cells against the effects of DNA damage, this has led to the proposal that changes in the status of p53 (de-regulation, mutation etc.) are indicative of worse prognosis. As a consequence, there is a plethora of small studies investigating the relationship between the p53 status of a tumour and clinical outcome. Some studies have used IHC others have used mutational analysis few have used both. Surprisingly, a comparison of a large

number of such studies revealed that assigning a significant prognostic value to p53 status is still not possible despite the enormous effort made to address this issue. This question is important for the clinical treatment of cancer. If p53 staining reveals a certain pattern (i.e. increased p53 in the nucleus), chemotherapy might be strongly advised because the particular tumour may be more aggressive and chemotherapy expected to provide a significant benefit to the patient. However, based on the comparison of a large number of studies, there is poor agreement between mutations in p53 and changes in its detection by IHC, even when the same tissue samples were used. Based on these findings, it is difficult for clinicians to rely on information obtained from IHC for p53 to make decisions about the most suitable therapy. Ultimately, it is the goal of such studies to provide a means to identify patients that will benefit from chemotherapy and to avoid treating those for whom the benefits are too small to justify the severe side effects. These findings further illustrate the need to establish standard procedures that permit determining the status of cancer-associated proteins reliably.

#### Background

[Mogensen M.M. et al. The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays of microtubule bundles in highly polarized epithelial cells. J Cell Biol. 157:1041-8 \(2002\).](#)

## IHC for $\beta$ -catenin

### Demonstration – MB

- Deparaffination, xylene 10'
- Hydration, 100, 96, 80, 70, 50 % EtOH 2' each
- Demi-water
- Blocking endogenous peroxidase with a PO-Blocking\* buffer for 15'
- Demi-water 2'
- Antigen retrieval boiling 20' in Tris-EDTA pH 9.0\*\*
- Washing 3 x in PBS 2' each
- Pre-blocking for 30' with 1% BSA/PBS
- Incubation 1st antibody 1:100 in (Pre-)blockingbuffer\*\*\* 2 h
- Washing 3 x in PBS 3' each
- Incubation 2nd Antibody (DAKO) Envision anti- Mouse-PO 1h
- Washing 3 x in PBS 3' each
- Incubation with DAB-H<sub>2</sub>O<sub>2</sub> 10'
- Washing with demi-water
- Mayer's Haematoxylin counterstaining 2'
- Tap water 3-5'
- Dehydration, Xylene
- Enclosure with pertex

\* PO blocking buffer 10 L (Stock):

- 83.2 g Citric Acid
- 215.2 g Na<sub>2</sub>HPO<sub>4</sub>\* 2H<sub>2</sub>O
- 10 L Demi-water
- 20 g NaAzide
- Preparing solution before use:
  - 190 ml Stock solution
  - Add 10ml H<sub>2</sub>O<sub>2</sub> (30%)

\*\* Tris- EDTA pH 9.0 10L:

- 12 g Tris
- 3.7 g EDTA
- 10 L Demi-water

\*\*\* 1<sup>st</sup> Ab =  $\beta$ -catenin from Transduction laboratories (Cat. No. 610154 250ug/ml)

## Supplemental protocols

### Fecal PCR assay for *Helicobacter*

Cheri Pasch and Ryan Burch (Dove lab)

#### *DNA isolation from fecal pellets*

##### Collection

Collect 3 pellets in empty 1.5 ml Eppendorf tube from bottom of cage for each cage to be tested. Pellets from 10 cages may be pooled into a single sample (use 15 ml tube for collecting pools. Further "pool" protocol info in parentheses).

##### Collection procedure

- Fill beaker ½ full with Clidox. Submerge tips of 2 pairs of forceps.
- Remove first pair of forceps, blot dry and check for pellet particles in teeth of forceps.
- Collect pellets from single cage. Check forceps and wipe off after each collection.
- Place "used" forceps in Clidox beaker and remove second pair for next cage.
- Repeat.
- Continue switching forceps between cages.

##### DNA isolation procedure

- Before starting you will need:
  - 1 tube/sample with 110 µl PBS
  - 1 Qiagen spin column and 2 collection tubes/sample
  - 1 tube/sample for final collection
- Add 1 ml PBS to each tube (10 ml for pools)
- Place samples in fridge for 1 hr to O/N to soften.
- Mash pellets using disposable Kontes pestle to form a slurry. Use a new pestle for each sample. (Use metal spatula for pools, using a new spatula for each sample.)
- Vortex THOROUGHLY!
- Add 500 µl PBS (5 ml for pools). Invert or vortex briefly to mix.
- Spin 5 minutes @ 700 x g.
- Remove 90 µl supernatant and add to 110 µl PBS in pre-prepared tube.
- At this point, you will use the Qiagen Blood and Body Fluid protocol to finish the isolation.

*PCR assay*

From Beckwith et al., Fecal PCR Assay for Diagnosis of *Helicobacter* Infection, J. Clin. Microbiol. 35, 1620-1623 (1997)

Primers: 5'-TATGACGGGTATCCGGC-3' and 5'-ATTCCACCTACCTCTCCCA-3'  
(from 16S rRNA gene conserved among members of the *Helicobacter* genus).

PCR mix  $\mu$ l per reaction

DIW	12.75
10X buffer	2.5
25mM MgCl <sub>2</sub>	1.5
10mM dNTPs	0.5
20 $\mu$ M primer1	1.25
20 $\mu$ M primer2	1.25
Taq	0.25
Qiagen fecal DNA	5.0

PCR profile

1. 94° x 30"
2. 94° x 2"
3. 53° x 2"
4. 72° x 30"
5. 44 x step 2
6. 4°

Run on agarose gel (2%) to yield 375-bp band in positive sample.

## Protocol for Generating Aggregation Chimeras

Cheri Pasch, Dove lab

(Sample images are available at <http://mcardle.oncology.wisc.edu/dove/Protocols.htm>)

### Day 1 (Sun)

1. Inject up to 18 B6 female donors or 12 B6 female donors and 12 B6 Tyr<sup>-/-</sup>Apc<sup>+/+</sup> female donors (age between 23 and 27 days, 14-16 grams) with 0.1 ml PMS (50 I.U. /ml) at 5:00pm (4:30-5:30pm OK). PMS is prepared in 0.9% NaCl and stored as aliquots at -20°C for 3 months.

### Day 2 (Mon)

Do nothing.

### Day 3 (Tues)

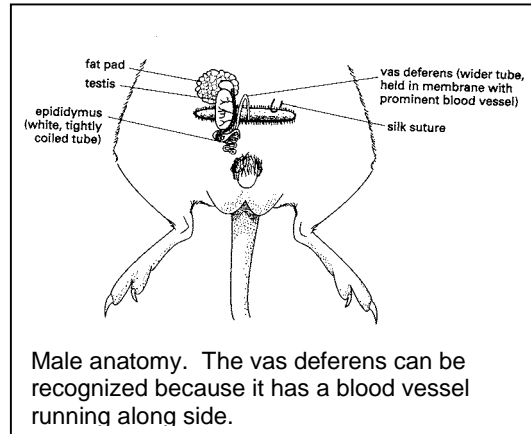
1. (We were having a problem with implanted females raising their litters and thus began using foster mothers. This may or may not be a necessity in increasing survival of chimera pups.) Foster chimera implants using ICRs. If the chimeras are not Tyr<sup>-/-</sup>, then put the pregnant implanted ICR into an ICR foster mother cage that has already dropped. If the chimeras are Tyr<sup>-/-</sup>, then do not put the pregnant implanted ICR into a foster mother cage; wait until the litter drops and foster at that time (tail clip non-pigmented chimera pups). Be sure to include ALL relevant info on foster mom's card.
2. Inject female donors with 0.1 ml HCG (25 I.U. /ml) at 5:00pm (~48 hours post PMS injection). HCG is prepared as a 250 I.U. /ml stock in 0.9% NaCl and stored as concentrated 100 µl aliquots at -20°C for 3 months. It is diluted 1:10 with 0.9% NaCl before injection.
3. Mate six of the B6 female donors to six B6 Apc<sup>1638N/+</sup> males or the six to twelve B6 Tyr<sup>-/-</sup> female donors to six to twelve B6 Tyr<sup>-/-</sup> Apc<sup>Min/+</sup> males. Mate the other six to twelve B6 female donors to six to twelve B6 Apc<sup>1638N/+</sup> Rosa 26/+ males.

### Day 4 (Wed)

1. Check yesterdays fostered implants to see if they have dropped. If so, then remove and discard the implanted ICR and the appropriate number of ICR pups.
2. Pull B6 females donors that have vaginal plugs. Put the females that were mated to the B6 Apc<sup>1638N/+</sup> males or the B6 Tyr<sup>-/-</sup> Apc<sup>Min/+</sup> males in one cage and the females that were mated to the B6 Apc<sup>1638N/+</sup> Rosa 26/+ males in another cage. Separate or discard females that were not plugged.
3. Set up ICR females with vasectomized ICR males. We choose up to 12 females that are in proestrus (red, swollen, moist vaginal area) and mate them 1x1 to

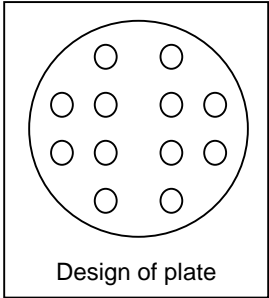
males to ensure that we obtain enough pseudo pregnant ICR recipients. Males must have been vasectomized at least 10 days before mating and can be used for several months.

- a. Anesthetize male.
- b. Shave abdomen of mouse.
- c. Open the body wall by cutting skin with dissection scissors, making a tiny (0.75 cm) incision about 1 cm above the genitals.
- d. Make a small incision in the body wall.
- e. Locate the bladder and gently fold back onto a sterile gauze pad using a surgical spear.
- f. The vas deferens tubes will be visible on either side.
- f. Grasp one vas deferens with a forceps and cut it close to the bladder.
- g. Holding the loose end of the vas in your forceps, gently tease away the blood vessel and fat that runs the length of the vas.
- h. Make a second cut near the connection to the epididymus and remove the cut section. As long as you take a large enough segment, the two ends should not re-anneal.
- i. Repeat with the other vas.
- j. Gently return the bladder to the body cavity.
- k. Close body wall with absorbent suture such as Vicryl.
- l. Close incision with wound clips.



### Day 5 (Thurs)

1. Pull ICR females that have vaginal plugs.
2. Prepare media:
  - a. If needed, open a new bottle of M2 (Sigma M-7167) or M16 (Sigma M-7292) media, syringe filter, and aliquot into 15ml (for M2) or 5ml (for M16) sets. Media is stored until the expiration date on the original bottle. We have tried alternate sources of media but found that the embryos did not develop as well.
  - b. Add antibiotics and BSA to M2 and M16
    - i. If needed, prepare a 100x antibiotic stock consisting of 10,000 units/ml penicillin G (Sigma P-4687) and 5,000 µg/ml streptomycin sulfate (Sigma S-1277). Aliquot into 250µl and store at -20°C indefinitely.
    - ii. Add 50 µl of this 100x stock to 5 ml of M16 and 150 µl of 100x stocks to 15 ml of M2. Invert tubes to mix.

- iii. Add 20 mg BSA to the M16 and 60 mg BSA to M2. Allow to dissolve (~5 minutes). You may place in 37°C incubator or leave at RT. Label “Pen-Strep + BSA” along with the date, and store at 4°C for one week.
3. Prepare M16 plates (for generation of aggregation chimeras):
- a. Place twelve 40 µl drops of M16 on ten Fisher (08-757-13A) 15 x 60 mm tissue culture plates (two rows of 4 in the middle, a row of 2 on each side).
  - b. Make a well in each drop by spinning a titanium-tipped pen (or Hungarian needle) into the plastic (I put a dab of mineral oil on my glove to help it spin more easily, and spin about 12 times while pressing down). This works best if you flame the pen, do one plate, wipe the pen and re-flame.
  - c. Overlay with mineral oil saturated with PBS (1:1 ratio of each – mineral oil is on the top). Oil is filtered prior to use with 0.8 µm filter units.
  - d. Incubate plates at 37°C in 5% CO<sub>2</sub> humidified incubator for at least 12 hours.
- 
- The diagram shows a circular plate with 12 wells. The wells are arranged in two rows of 4 in the middle, and a row of 2 on each side. The caption below the diagram reads "Design of plate".
4. Prepare glass pipettes. Glass pipets need to be siliconized to prevent embryos from sticking. Heat siliconized pipettes over an open flame and draw out ends using a tweezers to reduce the internal diameter. The opening of the glass pipet should be about twice the size of an embryo.

#### Day 6 (Fri)

1. Prepare plates at least one hour before generating chimeras and store at 37°C in 5% CO<sub>2</sub> (Label all dishes):
  - a. Two 20 x 100 mm plates (Falcon #35 3003) containing 15 ml PBS.
  - b. Three 10 x 35 mm (Falcon #35 3001) plates containing 2 ml of M2.
  - c. Two 10 x 35 mm (Falcon #35 3001) plates containing 2 ml Tyrode's solution (available from Sigma; prealiquot and freezer in 3 ml aliquots at -20°C). (Just dump into plate)
  - d. Five 15 x 60 mm plates (Fisher 08-757-13A) containing five 50 µl drops of M2.
  - e. Leave remainder of M2 media in incubator for use the next morning.
2. Set up stations for embryo harvest/chimera generation. Each microscope should have a p10 pipetman, a marker, a sharp tweezers (Dumont #5), and a syringe with a 30-gauge needle attached (all set on a Wyp-all) as well as the dissection tools (scissors, forceps, EtOH, bag and a WypAll).



3. Isolate embryos from female donors from the first strain.

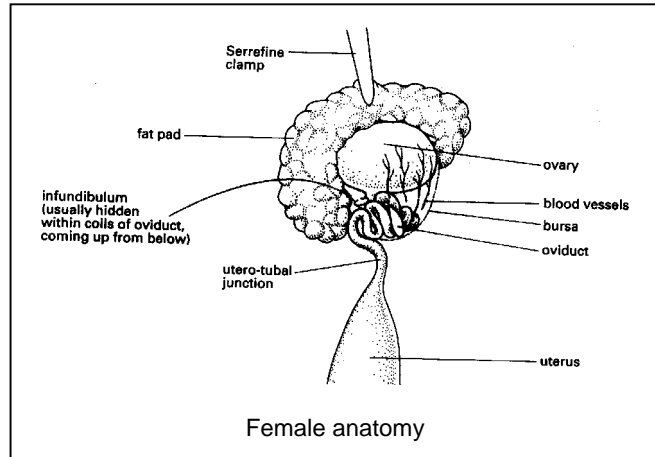
- a. Sacrifice female donors mated to B6 *Apc*<sup>1638N/+</sup> males or B6 *Tyr*<sup>-/-</sup> *Apc*<sup>Min/+</sup> males by CO<sub>2</sub> asphyxiation.

Remove uterus and ovaries and place in dish containing PBS.

- b. The following manipulations are performed under a dissecting microscope.

- c. Cut off each ovary at the junction with the oviduct. Make a second cut to remove all but 5 mm of the uterus. Place oviduct in dish containing M2.

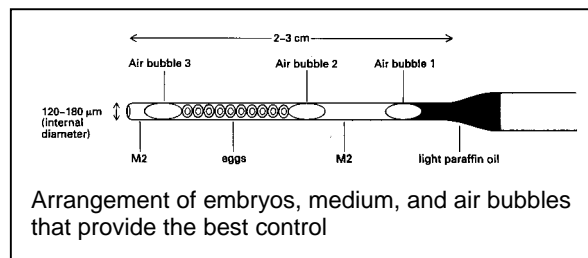
- d. Blow embryos out of the oviduct. Attach a 30-gauge needle to a 1 ml syringe and fill with 500 μl of pre-equalized M2. Hold the infundibulum with dissecting forceps and insert the 30-gauge needle. Flush out the embryos. If done correctly, the oviduct should expand and a stream of embryos should flow out of the uterus. Locate embryos under high magnification. Transfer 4 to 16 cell embryos to a plate containing five drops of M2 using a p10 pipetman or glass pipet.



4. Treat embryos with pre-equalized Tyrode's solution to remove the zona pellucida.
- Transfer a few embryos from M2 into Tyrode's solution using a pipetman.
  - Move embryos around in order to dilute M2 in which they were transferred.
  - Remove embryos as soon as the zona pellucida disappears.
  - Transfer embryos to a plate containing five drops of M2.
  - Wash embryos by moving them through the additional drops of M2.

5. Repeat all steps with female donors that were mated to B6 *Apc*<sup>1638N/+</sup> *Rosa* 26/+ males (the second strain).

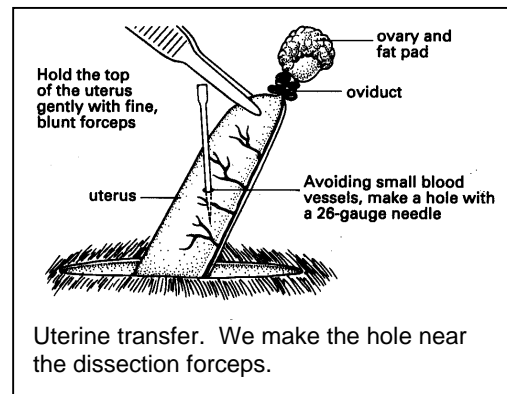
6. Generate aggregation chimeras:
- Transfer embryos using a glass pipette from M2 to M16 plates with wells such that there is one embryo from each strain per well.



- Make sure that embryos are touching in the wells and be careful not to jar the plate when placing in the 37°C incubator.

### Day 7 (Sat)

1. Blow aggregation chimeras out of the wells and transfer to a 10 x 35 plate containing 2ml of equilibrated M2.
2. Anesthetize pseudo pregnant ICR recipient using 0.01 ml Nembutal (pentobarbital) /g body weight.
3. Shave lower back.
4. We place the recipient on a warming pad during the implantation though this may not be completely necessary.
5. Make an incision through the skin and locate the ovary, which will be bright red.
6. Make a second incision through the body wall.
7. Pull the fat pad attached to the ovary out and place on a small piece of sterile gauze. Attach a small clamp to the fat pad to secure the ovary in the correct orientation.
8. Grasp the uterus near the top with a pair of dissecting forceps and puncture it with a 25 gauge needle attached to a syringe. Make sure the needle passes through the uterine wall.
9. With the forceps as a landmark, find the hole and transfer 6 to 8 embryos from M2 to the uterus using a glass pipette.
10. Slide the ovary and uterus back into the recipient and close the incisions with absorbent suture and wound clips. Alternatively, the body wall and skin can be closed simultaneously with wound clips alone.
11. Inject the recipient with 0.05 ml buprenorphine.
12. Wrap the recipient in a WypAll to conserve body heat.



### Day 8

Check on ICR recipient.

### Day 19

Remove wound clips.

### Day 24-26

Aggregation chimeras will be born 17-19 days post surgery. Pups must be fostered as mothers often kill their pups.

### Notes

1. Superovulation is done in order to set the timing of the experiment rather than to increase the yield of embryos. Females must be treated with hormones between 21 and 28 days of age before they begin their own cycle. The amount of hormone that is effective is dependent upon strain. We use 5 I.U. of PMS and 2.5 I.U. of HCG for B6 females.
2. Injection of hormones needs to be intraperitoneal.
3. BSA must be added to media to prevent embryos from sticking to the plates.
4. Minimize the time that embryos and media spend out of the incubator.
5. M2 is more stable than M16 at room temperature.
6. Extended treatment of embryos with Tyrode's solution will kill them, so treat only a few embryos at a time.
7. Aggregation chimeras must be blown out of the wells before they progress to blastocysts as it is impossible when they reach this stage of development.
8. Inject first bubble and embryos into the uterus. Limit the amount of air as it can interfere with implantations.
9. Sterile technique should be used for all surgeries.

Supplies for generating aggregation chimeras

<i>Item</i>	<i>Amount</i>	<i>Source</i>	<i>Cat #</i>	<i>Cost</i>
Pregnant Mare Serum (PMS)	1000 I.U.	Sigma	G-4877	\$52.30
HCG (Human Chronic Gonatrophin)	10000 I.U.	Sigma	CG-10	\$58.70
M2	50 ml	Sigma	M-7167	\$15.85
M16	50 ml	Sigma	M-7292	\$13.15
BSA	10 g	Sigma	A-3311	\$61.90
Mineral oil	500 ml	Sigma	M-8410	\$19.30
Tyrode's solution	100 ml	Sigma	T-1788	\$19.20
Penicillin G	1,000,000 U	Sigma	P-4687	\$11.50
Streptomycin sulfate	5 g	Sigma	S-1277	\$14.60
20 x 100 mm plates	Case of 500	Fisher	08-772E	\$209.40
15 x 60 mm plates	Case of 500	Fisher	08-757-13A	\$133.90
10 x 35 mm plates	Case of 500	Fisher	08-772A	\$209.00
Forceps, microdissecting	1	Roboz	RS-5130	\$37.20
Scissors, microdissecting	1	Roboz	RS-5910	\$59.50
Forceps, Dumont #5	1	Roboz	RS-5045	\$28.60
Clamp	1	Roboz	RS-5471	\$64.40
Cellulose surgical spear		Braintree Scientific	SP-40410	\$19.00
Disposable 1ml syringe				
30 ½ G needle				
Aggregation needle		BLS Ltd.,	DN-09	
See <a href="http://www.bls-ltd.com">http://www.bls-ltd.com</a>		Hungary		

## **BrdU labeling**

RH

Studies of proliferation in the mucosal tissues is of great importance in that many diseases that are associated with the intestine arise due to alterations in the rates of cell proliferation within the intestine. There are various approaches that can be used to study the cell kinetics of the small and large bowel such as <sup>3</sup>HTdR or BrdU labelling, or Ki67 and PCNA staining. The latter is not to be recommended since result can be highly variable and the protein is present through much of the cell cycle. BrdU and thymidine labeling generally tend to be the most reproducible and consistent means of assessment. BrdU is a derivative of deoxyuridine that is specifically incorporated into the DNA of proliferating cells during the S phase of the cell cycle (Chwalinski et al. 1988). BrdU labeling is a method that allows the study of cell kinetics via visualisation and analysis of the spatial distribution of DNA synthesizing cells in the colon and small intestine, and the frequency at which these cells occur. It is a means of examining the labeling Index (LI%) at each cell position, the data that can be analyzed using the program New Crypts, and is a suitable alternative to Thymidine labeling and produces similar results (Kellett et al. 1992).

### *Bromodeoxyuridine Staining Using the Boric Acid Buffer Method*

1. Dewax slides in xylene overnight.
2. Air-dry and mark around tissue with a PAP (hydrophobic) pen.
3. Wash three times in absolute alcohol for 5 minutes each.
4. Block endogenous peroxidase activity with 1% Hydrogen Peroxide for 30 minutes (diluted in Methanol).
5. Place slides in 1N HCL at 60 C for 8 minutes.
6. Neutralize slides in Boric acid buffer for 6 minutes.
7. Wash in PBS for 10 minutes. Repeat this 3 times.
8. Block with normal rabbit serum 1/20 for 30 minutes.
9. Tap off serum and add primary antibody rat anti BrdU 1:5 (Sera labs Mas 250b) for 1 hr.
10. Wash for 5 minutes in PBS, 3 times.
11. Add secondary antibody rabbit anti rat peroxidase (Dako P0162) 1:100 in 10% normal mouse serum for 1 hr.
12. Wash for 5 minutes in PBS, 3 times.
13. Immerse slides in DAB for 6minutes
14. Rinse in distilled water.
15. Place into 80% Methanol for 10 minutes and then into Thionin for 6 minutes.
16. Dip slides 5 times in 2 changes of 95% alcohol and then place in absolute alcohol for 1 minute.
17. Place in Xylene for 10 minutes and then mount slides.

### *Boric Acid Buffer*

Stock A: 0.2M Orthorbic Acid Solution (BDH 1005845) (7.44g in 600ml distilled water).

Stock B: 0.05M Sodium Tetraborate (Borax) (BDH 30717) Solution (9.5g in 500ml distilled water).

To Make Buffer add 550mls of Stock A with 450mls of Stock B and adjust pH to 8.4.

### References

1. Chwalinski S, Potten CS, Evans G. Double labelling with bromodeoxyuridine and [3H]-thymidine of proliferative cells in small intestinal epithelium in steady state and after irradiation. *Cell Tissue Kinet.* 21:317-29 (1988).
2. Kellett M, Potten CS, Rew DA. A comparison of in vivo cell proliferation measurements in the intestine of mouse and man. *Epithelial Cell Biol.* 1:147-55 (1992).

## **Apoptosis TUNEL assay**

ApopTag® RED IN SITU APOPTOSIS DETECTION KIT  
from Serologicals Corporation (<http://www.chemicon.com/webfiles/PDF/S7165.pdf>)

## **ISH: Non-radioactive *in situ* hybridization of paraffin embedded tissues**

Updated by Xiaodi Chen and Bill Ehrhardt 2/13/03

Modified from Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J. & Kern, S. E. *Cancer Res.* 62:5351-5357 (2002)

### ***Part A: Making Digoxigenin-labeled Riboprobes***

1. PCR is used to generate 300-800 bp sized DNA templates for antisense or sense riboprobes by incorporating the following T7 promoter into the 5' end of the antisense or sense primer.

5' CTAATACGACTCACTATAGGG 3'

*In a typical reaction using an amplified DNA template (e.g. purified PCR product, IMAGE clone, etc.) sufficient product can be derived from as little as three 15ul PCR reactions following 28 cycles. Run a small amount of PCR product on an agarose gel. If a robust PCR product is present and background bands are absent, gel purification is unnecessary.*

2. Use Qiagen PCR purification kit to purify the product.
3. Collect the product in 35µl of Tris buffer provided in the kit, measure DNA concentration using spectrophotometer, and dilute to 50ng/ul.
4. In vitro transcription. At room temperature (RT) mix the following reagents which can be obtained from Boehringer Mannheim:

dH2O water (Gibco-BRL ultrapure brand)	4.5 µl
100ng DNA template (50ng/ul)	2 µl
10X DIG RNA Labeling Mix (cat# 1277 073)	1 µl
10X transcription buffer (cat# 1465 384)	1 µl
RNAse inhibitor (cat# 799017)	0.5 µl
T7 polymerase (cat# 881 775)	1 µl

*DNA templates made with the T7 promoter on the original reverse primer will generate antisense probes, while those made with the T7 promoter on the forward primer will generate sense probes.*

5. Incubate 2 hours at 37°C.
6. Add 1ul DNase I, RNase-free (cat# 776 785) and incubate at 37°C a further 15 min.
7. Add 2ul 0.2M EDTA and place on ice.



8. Dilute to 100ul with DEPC water.
9. Purify the probe with BM QuickSpin column for RNA (we find that a second spin prior to loading sample onto column is required to remove excess buffer).
10. Measure the RNA concentration.
11. (*Optional*) Determine the amount of digoxigenin incorporation into the newly made riboprobe with Boehringer Mannheim Nucleic Acid Detection Kit (cat # 1175 041).

*Riboprobes are very stable, and once made can be stored for months to years at -80 °C. However, if RNA integrity is ever in question, verify RNA integrity and concentration by running 5ul (1ug) of RNA on a 6% TBE-urea gel alongside known concentrations of Marker (e.g. RNA Century-Plus size markers, Ambion cat# 7145).*

### ***Part B: In Situ Detection***

#### **Day 1**

*The following steps are carried out in 50 ml conical tubes. Each 50ml tube can fit 2 outward-facing slides. 30ml of solution is sufficient to immerse sections.*

*Prior to starting dewaxing steps, prewarm citrate buffer to 95 °C for Step #6. Also, preheat hybridization ovens to chosen hybridization temperature (see step 15).*

*All buffers and solutions used on Day 1 must be RNase free.*

1. Have your paraffin embedded sections cut onto microscope slides that are polylysine coated or charged. Once cut, these sections can be stored in any clean, dry container until ready for use.
2. Incubate slides in xylene 2 x 5 minutes.
3. Incubate in 100% ethanol 5 minutes.
4. Incubate in 70% ethanol 5 minutes.
5. Incubate in 50% ethanol 5 minutes.
6. Rinse in dH<sub>2</sub>O twice, then put into pre-warmed citrate buffer (0.01M, pH 6.0, fresh), incubate at 95°C for 30 min.
7. Cool down the slides to room temperature in the same buffer.
8. Incubate 5 minutes in TBS.

9. Immerse sections in Proteinase K (see stocks) diluted in fresh TBS (started from 5 $\mu$ g/ml). Incubate 5 minutes at room temperature.

*The following paragraph describing optimization of the Proteinase K digestion was included in the original protocol. We did not have great success with this technique, so we added Step #6 to lyse cells. We still used the Proteinase K digestion as described in Step #9, but we have not found it necessary to optimize conditions as described in the following paragraph. If, however, you were to experience background problems, the Proteinase K step could be reevaluated.*

*The optimal concentration of Proteinase K to be used for a run of in situ experiments using a particular probe must be determined, but a good starting point is to try 10-20  $\mu$ g/ml Proteinase K in TBS. Overdigestion of tissues can result in high background, while underdigestion results in no or very weakly detectable signal at the end of the experiment. In addition, addition of glass slides to prewarmed TBS usually lowers the temperature several degrees. Therefore, the incubation must be timed for 30 minutes once the temperature reaches 37 °C inside the conical tube. (Check the temperature inside the conical tube with a thermometer periodically. Alternatively, I find that it usually takes 10 minutes for the temperature to come back up to the incubation temperature once the slides are added to the TBS. Therefore, I just add 10 minutes to my incubation time for a total of 40 minutes).*

10. Rinse in TBS twice, then incubate in 3% hydrogen peroxide 15 minutes RT (made by diluting 1:10 commercial 30% Hydrogen Peroxide with TBS).
11. Immerse slides into 0.1M triethenolamine solution, add acetate anhydrate to final concentration of 0.25% and mix the solution well immediately. Incubate for 10 minutes at RT.
12. Place slides in TBST at RT.
13. In a 1.5ml RNase-free microfuge tube add digoxigenin-labeled riboprobe to fresh hyb. solution (DAKO, cat #S3304) (final concentration 100-500 ng/ml) ensuring a dilution of at least 1/50. Vortex.
14. Briefly dry the slide around the tissue with KimWipes. Put the HybriWell hybridization chamber onto the slide, firmly press over adhesive areas of hybridization chamber while making sure not to touch the tissue (normally two chambers for two tissue sections on a slide). Carefully add 30-35 $\mu$ l of mixed probe from the hole of one side until the liquid reaches the other hole.
15. Denature probe at 68°C in an oven for 10 minutes and place immediately in a prewarmed wet chamber and incubate overnight in a hybridization oven at 15-25° below the hybridization temperature (HT) predetermined for the individual probe (This calculated temperature is the temperature at which 50% of the probe is hybridized):

$$HT = 41 + 0.41(\%GC) - 500/n$$

where %GC is the percentage of GC in the probe (e.g. 60) and n represents the length of the probe in bases (42°C is a good start point).

*If the riboprobe is not well labeled with digoxigenin, it may help to add more probe for a better signal. The best amount to add has to be determined on a set of sections hybridized with a range of probe concentrations diluted in hybridization buffer. However, if increased probe concentration does not help, the final signal can be increased with no noticeable increase in background by designing additional probes against nonoverlapping regions of the transcript and adding 100-200 ng/ml concentrations of these additional probes in the same hybridization reaction.*

*In contrast, for certain transcripts (particularly abundant ones) non-specific background due to the binding of riboprobe can be eliminated completely (without reducing the signal) by titrating down the concentration of probe.*

## **Day 2**

*We do not use RNase free buffers after step 2.*

1. Rinse excess probe from slides by incubating in 50ml tubes containing 30ml 2X SSC for at least 5 min in a 52°C water bath.
2. Rinse with 2XSSC, then incubate in 2XSSC containing RNase A (25µg/ml) at 37°C for 30 minutes.
3. Stringently wash slides with pre-warmed 1X SSC, 50% formamide DI in a 50 ml tube (cat# AB600 American Bioanalytical) twice at 15-20°C below the HT for 20 minutes each.

*Remember to allow the SSC/formamide solution to come up to the desired stringent wash temperature once slides are immersed before timing the incubation. This usually takes approximately 10 minutes, so I incubate for 30 minutes for my first wash, then for 20 minutes for my second wash. Alternatively, one can preheat all buffers for Day 2 to desired temperatures beforehand.*

4. Stringently wash slides with prewarmed Stringent Wash Buffer once for 20 min at 10-15°C above the hybridization temperature. Make this final wash solution using Dako's 50X Stringent Wash Concentrate (which also contains detergent and blocking agent) diluted 1:50 (found in DAKO GenPoint kit, cat # K0620).
6. Rinse twice with TBST for 3 min at RT.

7. Quickly dry around the sections with a Kimwipe. Next, circumscribe the outside edges of the slide with a heat-resistant pap pen (e.g. RPI's Super HT cat #195505). Before the sections dry, add 35  $\mu$ l of blocking buffer (see stocks). Incubate for 30 min at RT.

*Before using blocking buffer, be sure to add 1/20 dilution of rabbit immunoglobulin Fraction (DAKO cat #X0903). We use blocking buffer with rabbit immunoglobulin for all steps in which blocking buffer is used. Before dilution, all antibodies should be spun in a microfuge (e.g. 5 min. max. speed) to prevent precipitate-related background.*

8. Incubate sections with rabbit HRP-anti-DIG (Dako cat # P5104) diluted 1:30 - 1:100 in. Blocking buffer for 30 min at RT. We use a hybridization chamber for all incubation steps to ensure that sections do not dry out.
9. Wash 3 X 5 min with TBST with agitation (I use a platform shaker set to 2-3).
10. Add one drop of ready-to-use biotinyl-tyramide (from DAKO GenPoint kit) directly to slides. Incubate in DARK 15 min at RT. (Use an inverted ice bucket to accomplish this).

*Once biotin is deposited, sterile conditions are no longer necessary. Coplin jars for all washes are suitable from this point on.*

11. Wash 3 X 5 min with TBST with agitation.
12. Incubate sections with secondary streptavidin for 15 minutes at RT (from DAKO GenPoint Kit).
13. Wash 3 X 5 min with TBST with agitation.
14. Develop with DAB, made from diluting DAB concentrate 1:50 in the provided dilution buffer (from DAKO GenPoint Kit). Add ~35ul to each section.

*Color develops quickly with DAB. Monitor color development under a microscope, and be prepared to quickly dunk slides in a Coplin jar filled with water to stop reaction.*

15. In a Coplin jar, counterstain for 1-3 dips with Mayers Hematoxylin (Dako, cat # S3309). Rinse twice with dH<sub>2</sub>O.
16. Dehydrate sections in graded alcohols, 30 seconds each: 50% ethanol, then 70% ethanol, followed by 100% ethanol.
17. Place in xylene 1 minute.
18. Finally, place glass coverslip on a Kimwipe, add one drop of Permount (Fisher) to coverslip, invert slide and press section onto coverslip.

## Tips:

1. **Never let the sections dry out after adding probes.**

3. For an excellent review and trouble-shooting guide of in situ hybridizations, read: **Shrihari S. Kadkol et al., *In Situ Hybridization-Theory and Practice. Molecular Diagnosis* 4(3):169-183, 1999**, or call Dr. Kadkol directly in Gary Pasternak's lab (5-7402).

## *Stocks for In Situ Hybridization*

*All buffers on Day 1 are RNase free. To make RNase free buffer, we add DEPC to final concentration 0.1% to Stock Buffer below, let sit at RT overnight, then autoclave.*

1. **RNase Free Water:** We use distilled H<sub>2</sub>O. Add DEPC (diethyl pyrocarbonate) to final concentration 0.1%. Mix well by shaking, let sit at least 12 hours, then autoclave.
2. **TBS pH 7.5:** 100 mM Tris, 150 mM NaCl.
3. **TBST:** Use TBS (from #2) add Tween 20 to final concentration 0.1%.
4. **Proteinase K:** The stock solution is made by dissolving one bottle of 100mg Proteinase K powder in 5 ml of RNase free dH<sub>2</sub>O. Freeze small aliquots (e.g. 500  $\mu$ l) at -20°C. When ready to use, thaw aliquot and dilute to optimal concentration in 50ml tubes with 37°C prewarmed TBS.
5. **Tri E Buffer:** 0.1 M triethanolamine, adjust pH to 8.0 with HCl.
6. **Citrate Buffer:** Dissolve 2.1 g citric acid and 1 g NaOH in 1 L distilled H<sub>2</sub>O. Adjust pH to 6.0 with NaOH.
7. **20X SSC:** Dissolve 175 g NaCl and 88.2 g Sodium Citrate Dihydrate in 1 L H<sub>2</sub>O, adjust pH to 7.0.
8. **Blocking buffer:** First make 100mM Tris HCl (pH 7.5), 150mM NaCl, 1% blocking reagent (highly purified casein) from the Digoxigenin Nucleic Acid Detection Kit (Boehringer Mannheim cat # 1175 041). With pipetting and vortexing blocking reagent will dissolve at 55°C in <1 hour. Store at 4°C and use within a month (longer storage may lead to higher background). Immediately before adding to sections, spin rabbit immunoglobulin fraction (Dako cat # X0903) in microfuge and add 1/20 dilution to the blocking buffer. To remove any precipitated blocking reagent spin in tabletop centrifuge 5 min. Store at RT during assay. Note: Do not add sodium azide to blocking solution as this can inactivate HRP over long periods of time!

## **IHC using MOM kit and various vector substrates for peroxidase**

Dove lab

### *Antigen Presentation*

1. Incubate for 1 hr in xylene to remove wax.
2. Incubate for 10 min in 100% ethanol.
3. Rehydrate through ethanol series: 90, 80, and 70%. Incubate for 5 min in each solution.
4. Microwave for 25 minutes on high power in citrate buffer. Use as much citrate buffer as possible to assure the buffer level never falls below the top of the slides.
5. Cool in running water.
6. Wash for 5 min in DIW.
7. Wash for 10 min in PBS.

### *Antigen Detection*

1. Wipe out area around sections with a WypAll or cotton swab. Apply PAP around sections. Do not allow sections to dry out.
2. Block endogenous peroxidase activity by incubating for 5 min in 3% H<sub>2</sub>O<sub>2</sub>/H<sub>2</sub>O at RT. H<sub>2</sub>O<sub>2</sub> comes as a 30% stock.
3. Wash in PBS for 2 min (2x).
4. Incubate sections for 1 hr in working solution of MOM mouse Ig blocking reagent.
5. Wash in PBS for 2 min (2x).
6. Incubate sections for 5 min in working solution of MOM diluent.
7. Tap of excess MOM diluent, and add 1° antibody diluted in MOM diluent. Incubate for 30 min at RT. Make up ABC reagent during this step. It must sit for 30 min before use.
8. Wash in PBS for 2 min (2x)
9. Apply working solution of MOM biotinylated anti-mouse IgG reagent. Incubate sections for 10 min.
10. Wash in PBS for 2 min (2x).
11. Add ABC to all sections and incubate for 5 min at RT.
12. Wash in PBS for 5 min (2x).

### *DAB staining*

1. Develop in DAB solution for 5 min in the dark. Check for staining. Depending on how old the DAB is, it may take more or less time to develop.
2. Wash under tap water for 5 min.
3. Wash in DIW for 10 min.
4. Counterstain with a 1:3 dilution of hematoxylin/H<sub>2</sub>O. Usually 10 seconds is enough.
5. Wash under tap water until no color is lost.
6. Dehydrate through ethanol series: 70, 80, and 90%. Incubate for 5 min in each solution.
7. Incubate for 10 min in 100% ethanol.

8. Incubate for 30 min in xylene.
9. Coverslip.

*Vector NovaRED staining*

1. Incubate sections with the substrate at RT until suitable staining develops, which can take between 5-15min. Use in the dark.
2. Wash sections for 5 min in RO water.
3. Counterstain with a 1:3 dilution of hematoxylin/H<sub>2</sub>O. Usually 10 seconds is enough.
4. Wash under tap water until no color is lost.
5. Dehydrate through ethanol series: 70, 80, and 90%. Incubate for 5 min in each solution.
6. Incubate for 10 min in 100% ethanol.
7. Incubate for 30 min in xylene.
8. Coverslip.

*Vector VIP staining*

1. Incubate sections with the substrate at RT until suitable staining develops, which can take between 2-15 min. Use in the dark.
2. Wash sections for 5 min in RO water.
3. Counterstain in a 1:10 dilution of eosin for 5-10 seconds. Keep checking the negative controls for staining. Dilute the eosin in DIW.
4. Incubate in 95% ethanol for 15 sec. Make sure that all ethanol used after staining with VIP is fresh – nothing that was used during the original rehydration of the samples.
5. Incubate for 15 sec in 100% ethanol, followed by another 2 minutes in a second container of 100% ethanol.
6. Incubate for 6 min in xylene.
7. Coverslip.

*Solutions*

Citrate buffer

2.1 g Citric Acid  
1.0 g NaOH  
H<sub>2</sub>O to 1L

DAB solution

0.25 mg/ml DAB  
0.03% H<sub>2</sub>O<sub>2</sub>  
200 ml PBS

MOM Mouse Ig Blocking Reagent

2 drops of stock solution  
2.5 ml PBS

MOM Diluent

600  $\mu$ l protein concentrate stock solution  
7.5 ml PBS

MOM Biotinylated Anti-Mouse IgG

10ul stock solution  
2.5 ml MOM diluent

ABC reagent

2 drops A to 2.5 ml PBS. Mix.  
2 drops B to above solution. Mix.

Vector NovaRED

1. To 5 ml of RO water, add 3 drops of reagent 1 and mix well.
2. Add 2 drops of Reagent 2 and mix well.
3. Add 2 drops of Reagent 3 and mix well.
4. Add 2 drops of the hydrogen peroxide solution and mix well.

Vector VIP

1. To 5 ml of PBS, add 3 drops of Reagent 1 and mix well.
2. Add 3 drops of Reagent 2 and mix well.
3. Add 3 drops of Reagent 3 and mix well.
4. Add 3 drops of the hydrogen peroxide solution and mix well.



## Detecting $\beta$ -galactosidase Activity in Whole Mounts

Dove lab

(Sample images are available at <http://mcardle.oncology.wisc.edu/dove/Protocols.htm>)

### *Preparation and Staining of Tissue*

1. Remove intestinal tract and flush it with PBS.
2. Lay intestine out on bibulous paper.
3. Pin tissue and paper to paraffin wax.
4. Fix tissue in 4% paraformaldehyde for 1 hour at 4°C.
5. Wash tissue in rinse buffer for 30 minutes at room temperature. Repeat two times.
6. Stain for several hours or overnight at 37°C, according to the signal strength.
7. Fix tissue in 10% formalin for at least 12 hours at room temperature.
8. Wash and store in 70% ethanol.

### *Notes*

1. Do not wash tissue with ethanol when dissecting.
2. We pour paraffin into 25 mm x 150 mm plate that has been scratched extensively and allow it to cool at room temperature.
3. Other fixatives such as gluteraldehyde can be used but we have had the best results with 4% paraformaldehyde.
4. Fixation must be done at 4°C.
5. Xgal is light sensitive. We weigh out enough for each experiment and then cover it with nitrogen and store in the dark at -20°C.
6. Xgal dissolved in DMF at 40mg/ml is diluted to 1mg/ml in the staining. Xgal is available from United States Biological as a powder (#X1000) or dissolved in DMF (#1000-05).

### *Solutions*

#### 0.1 M Sodium phosphate buffer

115 ml 0.1 M Sodium phosphate,  
monobasic  
385 ml 0.1 M Sodium phosphate, dibasic

#### 4% Paraformaldehyde

20 g Paraformaldehyde  
100 ml PBS

Heat 65°C with stirring for 2-3 hours.  
Freeze 20ml aliquots.

Thaw an aliquot for each experiment.  
Add 80 ml PBS.

#### Rinse buffer

0.4 ml 1 M MgCl<sub>2</sub>  
2.0 ml 1% Sodium deoxycholate  
2.0 ml 2% IGEPAL  
196 ml 0.1 M Sodium phosphate buffer

#### Stain

50 mg Xgal  
2 ml DMF  
0.106g Potassium ferrocyanide  
0.082g Potassium ferricyanide  
48 ml Rinse buffer

Dissolve Xgal in DMF and then add to rinse  
buffer.

## **LOH/MOH assay from formalin fixed mouse tissue**

Larry Kwong and Jim Amos-Landgraf, Dove lab

### *Tissue Preparation*

#### I. Large, Pedunculated Colonic Polyps (assumed to be near-pure tumors)

1. Transfer formalin-fixed tissue to 70% EtOH after overnight fixation in formalin at room temperature.
2. Dry down 2-10mg of tissue in a speedvac drier
3. Isolate DNA using a QIAmp DNA Mini Kit (#51306)

#### II. Flat Colonic Polyps or Small Bowel Polyps

1. Section tumors that have been fixed in formalin and stored in 70% EtOH.
2. Microdissect tumor cells from 8-15 slides (depending on tumor size) with a scalpel.
3. Digest microdissected material with 40ng of Proteinase K in 40 $\mu$ l of Proteinase K digestion buffer (20mM Tris, pH 8.7, 0.2mM EDTA, 1% Tween-20).
4. Incubate at 37°C overnight
5. Spin 5 min. and remove supernatant to clean tube.
6. Add 5 $\mu$ l of Chelex-100 (in 10mM Tris, pH 8.0, 0.1mM EDTA) to supernatant, incubate at 37°C for 1 hour.
7. Boil for 5 minutes.
8. Spin and remove 30 $\mu$ l of supernatant to clean tube, avoiding the Chelex-100.

### *PCR*

#### I. "LSEQ" Primers (346bp amplicon centered around the Min polymorphism)

Sense primer: CGGAGTAAGCAGAGACACAA

Antisense primer: GGGAGGTATGAATGGCTGAT

#### II. PCR Mix (vary amount of DNA as needed)

ddH <sub>2</sub> O	8.6 $\mu$ l
10x buffer	2.5 $\mu$ l
MgCl <sub>2</sub>	1.2 $\mu$ l
10mM dNTPs	0.5 $\mu$ l
Primer 1	1.0 $\mu$ l
Primer 2	1.0 $\mu$ l
Taq	0.2 $\mu$ l
DNA	10.0 $\mu$ l
<b>Total</b>	<b>25.0<math>\mu</math>l</b>

### III. PCR Cycling Program

<i>Step#</i>	<i>Temp</i>	<i>Time</i>
1	94	3'
2	94	15"
3	57	2'
4	75	1'30"
Steps 2-4 30 cycles		
5	75	10'

#### *PCR Cleanup*

Cleanup can either be performed with a PCR purification kit (QIAGEN, Promega, etc.) or use of ExoSap (Exonuclease I with Shrimp Alkaline Phosphatase).

#### *Sequencing*

##### I. Sequencing reaction mix

DNA	~150ng
Terminator Big Dye Ver 3.1	2.0λ
5x Big Dye Buffer	4.0λ
Primer	2.0λ

Run on same PCR program as above.

##### II. Sequencing Cleanup

Purify the reaction on G-50 columns. Sequencing facilities generally take the sample as-is or dried down and resuspended in 4λ of 1:1 formamide:running dye.

#### *Reading the Sequencing Peaks*

Peaks can be read on freeware (e.g.: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) programs. Sequencing peak heights can be measured as an arbitrary fluorescence unit by a tool within the program.

$$\text{LOH ratio} = \frac{\text{peak height of the wild-type nucleotide}}{\text{peak height of the Min nucleotide}}$$

Values less than 0.5 are considered LOH; values exceeding 0.8 are considered MOH. Anything in between 0.5 and 0.8 or above 1.2 is considered to be either contaminated by non-polyploid tissue or a result of lab error and should be repeated.