Preface

The contents of *Colorectal Cancer: Methods and Protocols* aim to instruct investigators in all the key genetic, cellular, and molecular biological methods of analyzing colorectal tumors. The focused techniques and assays are described in sufficient detail to allow researchers to start an experiment on colon tumors and proceed from beginning to end as if the expert in the field who has performed these studies were guiding them at the bench. Of note, most of the chapters in this volume are written by those scientists who pioneered these methods and assays in their respective fields.

The chapters in *Colorectal Cancer: Methods and Protocols* describe "state of the art" methods to analyze colorectal tumors, ranging from gross microdissection of specimens to specific molecular analyses. Included are coverages of mutational assays, instability testing, immunohistochemical assays, chromosomal studies, and gene expression analyses. The goal of our volume is to facilitate the performance of colorectal tumor biological experiments by investigators at various levels of training—from graduate students and postdoctoral fellows to principal investigators who desire to advance our understanding of colon cancer development.

Recent advances in the fields of molecular genetics, signal transduction, DNA repair, and genomic instability—especially as they relate to colorectal tumorigenesis—make this comprehensive coverage of molecular assays of this cancer particularly timely. The initial section of the volume describes gross microdissection of colon tumors and the establishment of cell lines and xenografted tumors. The next section describes chromosomal analyses, including comparative genomic hybridization and FISH assays. Mutational analyses of colon tumors and of blood samples to determine whether they have inherited a significant predisposition for colorectal cancer development follows. Microsatellite instability testing is also presented. Gene expression analyses including immunohistochemical assays, Western blotting, and microarray assays are in the final section to complete the volume.

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Isolation of a Purified Epithelial Cell Population from Human Colon

James K. Roche

1. Introduction

While *in situ* techniques have been valuable in identifying the presence and localization of cytoplasmic and membrane components in tissue ***(1)***, there is often a need to study directly one or more cell types, free from its own microenvironment. For the human colon, isolation techniques to allow direct study have been described for mononuclear cells in the lamina propria, smooth muscle cells at or below the muscularis mucosae, and cells of the enteric nervous system, located between the subserosa and the lamina propria ***(2–4)***. More recently, interest has risen to isolate populations of intestinal epithelial cells, for investigations of human colonic adenocarcinoma—which originates from colonic epithelia; as well as for study of the epithelial response to infection and inflammation. The technique for isolating epithelial cells from the human colon involves mechanical dissection to separate mucosa from the muscle layers which are discarded; and enzymatic digestion of collagen, followed by discontinuous gradient centrifugation in Percoll. The goal is to isolate >90% pure epithelial cells. Although the cells appear intact under the microscope, viability is variable from 50–80%. The yield depends on the size of the available tissue.

2. Materials

2.1. Buffers

1. Hanks balanced salt solution (1X) without Mg^{2+} and Ca^{2+} (store at room temperature).
2. Dulbecco's phosphate-buffered saline (1X) without Mg^{2+} ad Ca^{2+} (store at room temperature).

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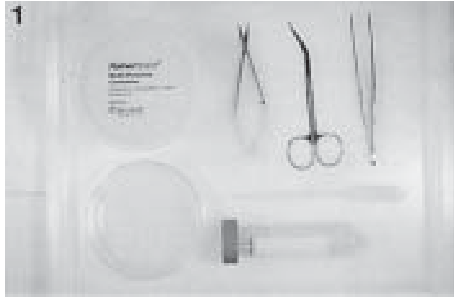

Fig. 1. Materials and instruments used for epithelial cell isolation. Moving clockwise from top left corner: surgical cutting board, small plastic container, surgical scissors, forceps, plastic transfer pipet, 50 mL centrifuge tube, and a Petri dish.

3. RPMI medium 1640 (1X), 0.1 μm filtered with L-glutamine (store at 4°C).
4. Percoll (sterile), 1000 mL, density 1.129 g/mL (store at 4°C).
5. 1 m*M* EDTA solution made in HBSS (store at 4°C).
6. 0.15% dithiothreitol solution made in HBSS (store at room temperature).
7. Trypan blue solution (*see* **Notes 1** and **2**).
8. HEPES buffer solution (1 *M*) (store at 4°C).

2.2. Chemicals

1. DNase enzyme (Worthington) (store at –20°C).
2. Dispase enzyme (Boehringer Mannheim) (store at 4°C).
3. Dithiothreitol DTT (Sigma) FW: 154.2.
4. Sodium chloride (Sigma) FW: 58.44.
5. Trypan blue (Kodak) FW: 960.81.
6. Thimerosal (Sigma) FW: 404.8.
7. EDTA disodium salt dihydrate (Sigma) FW: 372.2.

2.3. Equipment (see Note 3 and Fig. 1)

1. Fine tip transfer pipet (sterile).
2. Centrifuge tubes 50 mL.
3. One plastic surgical cutting board 12" × 12".
4. Forceps (small) (sterile).
5. Surgical scissors (sterile).
7. Flat bottom plastic disposable containers with tops.

3. Methods

3.1. Obtaining the Specimen

Obtain the surgical specimen. Select an appropriate area based on clinical diagnosis. Place tissue in 100 mL of ice-cold HBSS in a plastic disposable

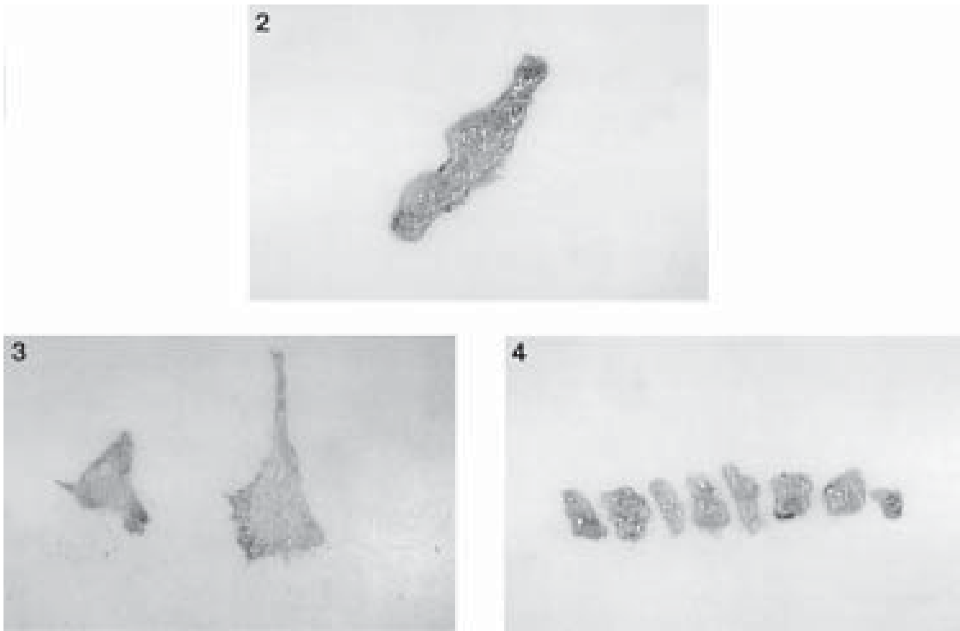

Fig. 2. Colonic specimen, shortly after surgical resection, opened longitudinally, with mucosal surface facing camera. To process this specimen, mucosa is stripped from the deeper muscle layers.

Fig. 3. Appearance of human colonic mucosa after it has been stripped from muscle and serosa. The next step is to cut the mucosa into 2 cm strips and then incubate with 0.15% Dithiothreitol/HBSS for 30 min to remove excess fat and other debris.

Fig. 4. Small pieces of colonic mucosa measuring 2 cm × 1 cm. The small sections will increase the surface area and allow more epithelial cells to be released from the tissue.

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container and transport immediately to the laboratory. Begin the procedure as soon as the specimen is acquired. Long exposure of tissue to outside environment reduces cell yield (*see* **Note 4**).

3.2. Preparing and Dissecting the Mucosa

1. Remove the specimen from HBSS. Place tissue on a flat surface (dissecting board) covered with dry paper towels and remove fat, necrotic tissue and gross debris (*see* **Fig. 2**).
2. Place slightly stretched tissue flat on paper towels, mucosal side up. Using curved fine forceps, gently pinch and lift the mucosa at one edge of the specimen. Cut between the mucosal and the muscle layers with fine curved iris scissors, starting at the lifted edge of the specimen and if possible, longitudinally to the circular

folds. Put the mucosal strips in a 100 mm Petri dish containing HBSS. Cut the strips approx 4 cm in length and 1–2 mm in width. Be sure that no muscle is included underneath each mucosal strip. When in doubt, invert strip, inspect it visually and remove any muscle inadvertently included (*see* **Figs. 3** and **4**).

3.3. Removal of Residual Mucus and Epithelial Cells

1. After complete removal of the mucosa, rinse strip thoroughly in a Petri dish containing fresh HBSS and transfer them to a flat bottom plastic disposable container with 50 mL of HBSS, 0.15% dithiothreitol and a magnetic stirring bar (*see* **Note 5**). Place container on a stirring plate at room temperature, put lid on and set speed at approx 0.30*g* for 30 min to dissolve residual mucus and free additional debris. At the end of the stirring period, the solution will be slightly cloudy and small floating debris is usually observed.
2. Remove the mucosal strips and the stirring bar, rinse them in a Petri dish with fresh HBSS and transfer them to a new container with 100 mL of HBSS, 1 m*M* EDTA, pH 7.2. Stir at room temperature for 60 min to releases epithelial cells from the basal lamina. The solution will become cloudy as the epithelial cells detach from the lamina propria. Stirring must be gentle, yet vigorous enough to keep all tissue floating in suspension, and not simply to push the strip around at the bottom on the container.
3. Repeat the 60 min stirring period once or twice depending on the conditions of the specimen (*see* **Note 6**).
4. Collect EDTA solutions in 50 mL centrifuge tubes. Spin down 470*g* for 5 min and resuspend in 15 mL RPMI media.

3.4. Isolation and Purification of Intestinal Epithelial Cells Using Dispase and Percoll

1. Add 45 mg Dispase and 15 mg DNase to the combined epithelial cells (final enzyme concentrations 3 and 1 mg/mL, respectively) and incubate in a 37°C water bath for 30 min. Vortex for 10 s at 5 min intervals. Use the minimum force required to vortex to minimize damage to cells. Small intestinal specimens almost invariably require longer stirring periods than large bowel specimens due to the release of many more epithelial cells from a comparable surface area as a result of the presence of villi. Specimens with mucosal inflammation will require variable times, depending on the degree of inflammation and the extent and severity of damage to the epithelial cell layer.
2. Spin the cells at 200*g* for 5 min, carefully discard the supernatant and wash again in HBSS. Following wash, resuspend in 5 or 10 mL RPMI depending on the size of the pellet.
3. Prepare an aliquot of cells for trypan blue staining and microscopic examination (*see* **Note 2**). The preparation should be a mixture of epithelial cells, mononuclear cells, and red blood cells. The preparation should be 95–100% viable and mostly single cells, any clumps containing 3–4 cells at most.

4. A 50% Percoll solution is used to separate epithelial cells from mononuclear and red blood cells. For most preparations 2 gradients are sufficient, however 1 or 4 gradients can be used with small or large preps. Gradients are prepared by mixing 10 mL Percoll with 10 mL PBS in 50 mL centrifuge tubes. Adjust the cell suspension volume to 5 mL per gradient and overlay each gradient with 5 mL of cell suspension. Centrifuge the gradients at 470*g* for 20 min.
5. The epithelial cells will equilibrate at the top of the Percoll layer, while the mononuclear cells and red blood cells will pellet at the bottom of the tube. Collect the epithelial cell layer in a 50 mL centrifuge *excluding* as much of the gradient material below as possible (*see* **Note 7**). Most of the epithelial cells can be recovered in 10–15 mL, leaving at least 10 mL in the gradient tube. Do not include material form the conical portion of the tube!
6. Dilute the recovered epithelial cells with RPMI and centrifuge at 830*g* for 5 min. Resuspend the epithelial cells in RPMI, spin at 470*g* for 5 min, then transfer to a 15 mL tube.
7. Resuspend in a volume appropriate for counting, and prepare an aliquot for trypan blue staining. Count live epithelial cells, live mononuclear cells and dead cells.
8. Final step: what to do with cells?
   a. Freeze cells at –80°C (*see* **Note 8**).
   b. Use cells in functional assay.

4. Notes

1. Please handle with caution! Thimerosal in powder form is toxic by inhalation, after contact with skin, and when swallowed. It is irritating to eyes, respiratory system, and skin. It is also a possible mutagen with target organs being kidneys and nerves. Wear suitable protective clothing, gloves, and eye/face protection when dealing with thimerosal as a powder. When thimerosal is dissolved, gloves are still recommended.
2. Counting solution: 45 μL (4.5% NaCl, 0.2% thimerosal).
   180 μL (0.2% trypan blue, 0.2% thimerosal).
3. Any equipment labeled "Sterile" means autoclaved individually wrapped to assure sterility.
4. When dealing with any human tissue, please use the utmost care to assure the safety of yourself and your lab. Dispose of anything that comes into contact with human tissue in your contaminated materials box. Isopropyl alcohol (70%) sterilizes everything.
5. Most of the solutions such as EDTA, 0.15% dithiothreitol, and trypan blue solutions can be made ahead of time.
6. A minimum of 2 EDTA incubations ensures higher epithelial cell counts.
7. When collecting the cells from the Percoll, use a fine tip plastic transfer pipet. When pipeting the cells in the centrifuge tubes, try not to make any bubbles. Bubbles may harm the epithelial cells.
8. At the end of the isolation, centrifuge the cells into a pellet and discard supernatant. Using a pipetman, place the cells in the cryogenic tube excluding as much of

the media as possible. Fast freeze the cells by placing the tubes in a small volume of liquid nitrogen. Label tubes with cell type, cell number, diagnosis, patient name/number, and so on.

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