Chapter 6

Ex Vivo Culture and Separation of Functional Renal Cells

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Abstract

The following methods outline the procedures for isolating primary renal cells from kidney tissue via enzymatic digestion, followed by their culture, harvest, and then fractionation of renal subpopulations from primary culture. The current methods describe procedures to sub-fractionate biologically active cells that have been used to treat and stabilize renal function in models of chronic kidney disease (Kelley et al. Am J Physiol Renal Physiol 299(5):F1026–F1039, 2010).

Key word Renal cell therapy

1 Introduction

The incidence and prevalence of CKD are rising worldwide and especially in the United States. New treatment approaches are required to improve quality of life among CKD and ESRD populations who currently have limited healthcare options. Previous methods have been successful in isolating kidney epithelial cells for regenerative medicine (1). Evaluation of a recently identified and novel renal cell-based therapy (4) may predict the potential impact of these therapies in CKD patients. Recent reports using fate mapping strategies identified the resident renal tubular epithelia, not a specialized progenitor cell (2), as the primary cell source responsible for repairing the kidney (3). Taking advantage of this fundamental learning of kidney repair and regeneration, we recently reported a novel cell-based treatment approach demonstrating the reproducible isolation and expansion ex vivo of a selected population of renal cells enriched for tubular epithelia (4). Herein we describe a method for the selection of a population of renal cells established to have reparative and regeneration ability to augment renal tissue function in animal models of CKD.

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2 Materials, Reagents, and Equipment

2.1 Materials

1. Freshly harvested kidneys collected not more than 1 day prior; perfused with phosphate-buffered saline (PBS) at the time of harvest and stored at 2–8°C in Viaspan.
2. Batch record or notebook.
3. Freezer box for sample storage and analysis.
4. Pipette tips capable of dispensing volumes ranging from 2–1000 μl.
5. Serological pipettes: 2, 5, 10, 25, 50 ml; aspirating pipettes.
6. Transfer pipettes.
7. Sterile 1.5 ml microcentrifuge tubes for RNA and Western Blot collection.
8. Non-sterile 1.5 ml microcentrifuge tubes for preparing cells for counting.
9. 15 and 50 ml conical bottom polypropylene tubes.
10. Pre-weighed 50 ml conical tubes for tissue digestion.
11. 1.5, 15, and 50 ml tube racks.
12. Sterile 150 cm dishes (or comparable) to hold instruments and for mincing kidneys.
13. Sterile 150 cm dishes pre-weighed for minced tissue.
14. 100 μm Steriflip® filters (Millipore, SCNY00100).
15. Fine-tipped forceps (curved or straight) (sterile).
16. Scalpel handle with appropriate blade or disposable scalpel (sterile).
17. Alcohol wipes.
18. Bench Wipes (paper towels).
19. Hemocytometer.
20. Bench Counter (for counting cells on hemocytometer).
21. Sterile Nunc T500 flasks (Item #132913).
22. 70% ethanol spray bottle.
23. Bacdown spray bottle.

2.2 Reagents

1. Wash buffer: DPBS calcium and magnesium free (Invitrogen Gibco 14190-235).
2. 50:50 growth medium 50% DMEM high glucose (4.5 g/l) (GIBCO 11995), 50% Keratinocyte-SFM (GIBCO 17005) containing human recombinant epidermal growth factor 1–53 (EGF 1–53), Bovine Pituitary Extract, 5% FBS (GIBCO 16000), 1× Anti–Anti (GIBCO 15240), and 1× Insulin Transferrin Selenium (GIBCO 41400).
3. Trypan Blue 0.4% (GIBCO 15250).

4. Digestion buffer: Dispase I 4 U/ml (StemCell Technologies 07913), 5 mM final calcium chloride (Sigma C2661), Collagenase type IV (Worthington 4212) reconstituted with dispase to a final concentration of 300 U/ml. Always check units on collagenase bottle b/c it changes with every lot (see Note 1).
5. Calcium chloride stock at 500 mM (100×). For 58 ml of final volume add 5.8 ml.
6. Hold digestion buffer at 37°C until ready for use.
7. 40 ml of digestion buffer is needed for each gram of tissue being digested.
8. Neutralization buffer: 5% FBS in DMEM (or comparable medium such as KGM) for neutralizing digestion buffer.
9. 30% Optiprep gradient medium (50% Sigma Iodixanol stk 60%w/v: 50% KSFM).
10. Keratinocyte-SFM.
11. 1× PBS (GIBCO 14190).
12. Liquid nitrogen for snap freezing.
13. 4% Paraformaldehyde for cell fixation (if needed for flow cytometry).
14. Freezing media (80% HTS-FRS, 10% DMSO, 10% FBS).
15. Sterile deionized (Di) H_2O for cleaning instruments.

2.3 Equipment

1. Biological safety cabinet (BSC)/tissue culture hood.
2. 37°C, 5% CO_2, humidified incubator.
3. Water bath 37°C.
4. Upright light microscope.
5. Centrifuge capable of 800 × *g* with appropriate bucket adapters for various tube sizes.
6. Tube rocker.
7. –80°C freezer.
8. Digital scale for weighing tubes and tissue.
9. Sonicator and milk bath for cleaning surgical instruments.
10. Pipettors (Ranin) capable of dispensing volumes ranging from 2–1000 μl.
11. Serological pipet controller (Drummond).

3 Methods

3.1 Renal Cell Isolation

1. Prior to kidney processing, clear the BSC and clean all surfaces of the cabinet with Bacdown (or sufficient substitute) followed by 70% ethanol or isopropyl alcohol.

2. Note catalog numbers and lot numbers of all reagents being used on batch record.
3. Prepare digestion buffer (see Subheading 2.2 for recipe) and hold at 37°C until use. Use estimate of tissue weight to determine the amount to prepare or wait until tissue weight is determined. 40 ml of digestion buffer required per gram of tissue to be digested.
4. Prepare hoods with necessary tools needed for procedure (surgical instruments (forceps, scalpels, and blades), plates, alcohol wipes, markers, tubes, pipettes, pipette tips, wash buffer (PBS w/o Ca^{+}, Mg^{+}), sterile microcentrifuge tubes).
5. Pre-weigh several 50 ml conical polypropylene tubes and record weight on tube. One tube will be required per gram of tissue to be digested. These weights will be used to check that 1 ± 0.1 g is added to each tube.
6. Pre-weigh two P150 dishes for weighing intact kidneys as well as kidney tissue prepared for digestion.
7. Prepare tube rocker by sanitizing with 70% ethanol and place in incubator to allow equilibration to 37°C.
8. Retrieve pre-flushed and cooled kidneys from Viaspan or other suitable storage solution. Verify that sample is cold (not frozen or warm) and verify integrity of packaging (no leaks, holes, etc.). Verify shipping sheet and sample contents match. On batch record, note the unique sample IDs and condition of sample (cold, warm, etc.), and indicate shipping/storage media (Viaspan) strain, and age of sample if applicable.
9. Spray primary container with 70% ethanol and wipe down.
10. Remove parafilm from around the mouth of the tube (if applicable), spray down with 70% ethanol, and wipe. Place tube in the BSC.
11. Aspirate the remaining shipping medium and wash the kidney twice with CMF-DPBS.
12. Transfer the kidney to pre-weighed P150 and weigh. Record the weight of pre-weighed dish, kidneys + dish, and determine the weight of the kidney tissue alone.
13. After washing, aspirate buffer, place tissue in dish or plate, and then remove and discard connective tissue, calyx, and capsule around the kidney using sterile forceps and scalpel.
14. Manually mince together the remaining kidneys using scalpel blade making finely chopped slurry of tissue. Kidney tissue pieces should be minced finely.
15. Add ~1.0 g (±0.1 g) kidney tissue to each pre-weighed 50 ml conical tube. Check weights of tube to confirm that weight of tissue within each tube is approximately 1 g (±0.1 g). Adjust tissue amounts if needed.

16. Add 20 ml of premade digestion buffer to each 50 ml conical tube containing ~1.0 g kidney tissue.
17. Digestion 1: Incubate digestion tubes at 37°C on a tube rocker located in the incubator for 20 min.
18. After 20 min, remove tubes from incubator/rocker and place in BSC.
19. Allow undigested tissue to settle to the bottom of the tube.
20. Aspirate supernatant from the top of each tube and discard (leaving only undigested tissue in the bottom of the tube, less than 5 ml). This helps to remove red blood cells, connective tissue, and other cell debris from sample.
21. Add an additional 20 ml of warmed digestion buffer to each tube containing undigested tissue.
22. Digestion 2: Incubate digestion tubes at 37°C on a tube rocker located in the incubator for 40 min.
23. After 40 min, remove tubes from rocker/incubator, combine the contents of two tubes to yield approximately 40 ml per tube, and run each sample through a 100 μm SteriFlip® filter using an aspirator.
24. Flow through will now be in a new, sterile 50 ml tube. This is an *unfractionated* sample that should be reserved.
25. Add 5 ml of neutralization buffer to each tube and mix well. Centrifuge at 300 × *g* for 5 min.
26. Aspirate supernatant and resuspend pellet in KSFM. Utilize sufficient volume such that a count can be made from this cell suspension. Typically a minimum of 10 ml per gram of tissue digested is required.
27. Remove small cell sample for counting using a hemocytometer (see Note 2). It is recommended to use a manual hemocytometer in order to obtain an accurate count during this step (see Note 3).
28. Record live cell, dead cell, cell viability, cells per ml, and total number of cells on batch record. Prepare samples of unfractionated cells for initial analysis (see Note 4).
29. For the portion of UNFX (unfractionated) cells that are to be cultured immediately, proceed as described below.
30. Based on the total remaining cell number in sample, calculate how many gradient tubes (15 ml tubes) are needed to load 60–75e6 cells/tube.
31. Pellet cells as described previously, 300 × *g* for 5 min, and wash once more with KSFM.
32. After wash resuspend cells to a final concentration of 10–15 × 10e6 in KSFM (see Note 5).

33. Establish 15% mixing gradient: Add 5 ml of 30% Optiprep to each 15 ml tube required for gradients.
34. Aliquot 5 ml of resuspended cells into each 15 ml tube.
35. Mix tubes by inversion six times.
36. Carefully layer 1 ml of PBS on top of Optiprep/cell mixture. (This will form a liquid barrier between the cell mixture and outside the air and will protect cells after centrifugation.) If 50 ml tubes are used, layer 5 ml.
37. Centrifuge at 800 × *g* for 15 min without brake (ensure that there is a balance tube in the centrifuge, if needed).
38. Once the centrifugation is complete, carefully remove tube(s) and make sure that there is a cell band located around the 9–10 ml mark and a pellet at the bottom of the tube.
39. Collect and combine all cell bands by aspirating via transfer pipette and transfer band contents into a new 50 ml conical tube. (Collecting 1 ml above or below the cell band is acceptable for accuracy.)
40. Aspirate out the remaining density medium and discard via vacuum aspiration (leaving the pellet in the tube).
41. Resuspend all pellets with KSFM.
42. Combine collected bands and pellets together adding enough KSFM to mixture to reach a minimum of 2:1 ratio KSFM to cell/Optiprep mixture. Sample may need to be split into multiple 50 ml conical tubes to achieve the 2:1 ratio.
43. Mix tubes by inversion six times (see Note 6).
44. Centrifuge at 300 × *g* for 5 min.
45. Aspirate supernatant via vacuum aspiration and discard (leaving pellet in tube).
46. Resuspend cell pellet with KSFM to desired volume. This is the initial 15% band + pellet (B + P) sample.
47. To establish cultures for scheduled use: Rat kidney cells are plated at 50,000 cells/cm^2 at isolation, typically in T500 with 150 ml of kidney growth medium per flask at 37° C/5% CO_2 under standard tissue culture (TC) oxygen. If however, the culture period is to be 4 days for scheduling purposes (3 days 21% O_2/1 day 2% O_2), cells are plated at 30,000 cells/cm^2.
48. To prepare cells for cryopreservation for future culture, harvest, and implantation or analysis, continue as described below.
49. Centrifuge collected volume containing the cell # desired to cryopreserve in 50 ml tubes, centrifuge at 300 × *g* for 5 min, and then wash pellet with unsupplemented KSFM. Centrifuge at 300 × *g* for 5 min.

3.2 Renal Cell Cryopreservation

1. Prepare cryopreservation media as described previously (FRS 80% vol, FBS 10% vol. and DMSO 10% vol). 1 ml of cryopreservation media is required per vial of cells to be frozen. Cells may be frozen at up to 50 × 10e6/ml.
2. After final wash is complete, aspirate supernatant. Tap tube containing pelleted cells at this point to resuspend the cells.
3. Add previously prepared cryopreservation media to the pellet in a dropwise manner until volume is equivalent to that required for freezing.
4. Transfer 1 ml of cells in cryopreservation media to each prelabeled cryovial. Tubes should be labeled with sample ID, cell number/vial, and date frozen.
5. Transfer labeled and filled vials to the rate-controlled freezer to freeze.
6. Once frozen, transfer to liquid nitrogen storage.
7. Retrieve all snap-frozen samples from liquid nitrogen and place in a labeled freezer box. Place box in −80°C freezer for analytical interpretation.
8. All cells should be distributed at this point to immediate culture, cryopreservation, RNA, Western Blot, or other analytic methods.

3.3 Renal Cell Culture and Harvest Procedure

1. Upon isolation of primary kidney cells, verify cell number.
2. Place 50:50 culture medium, trypsin, and KSFM in 37°C water bath to pre-warm.
3. For cultures to be harvested 3 days later, plate 50,000 cells/cm^2 in desired TC-treated vessel using complete 50:50 media at a volume that is recommended by vessel manufacturer (see Note 7).
4. Incubate cells for 48 h without disturbing in 150 ml total volume.
5. After 48 h (day 2 postseed) change medium to remove unattached cells and debris.
6. Replenish flasks with 100 ml of fresh medium.
7. Switch all culture vessels to 37°C, 5% CO_2, low O_2 (2%), humidified incubator.
8. Maintain cultures at low oxygen O_2 (2%) for 18–24 h.
9. On the day of harvest (day 3 postseed) monitor confluency of cells via light microscopy and image to document morphology and confluency and record on batch record or notebook. Cells should be approximately 60–80% confluent at this point (Fig. 1).

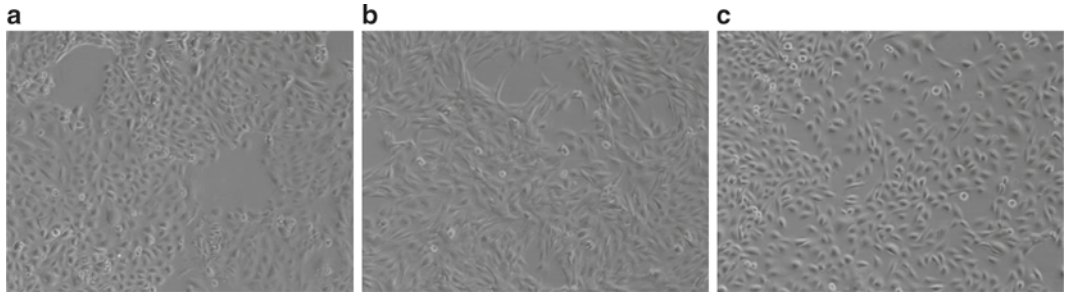

Fig. 1 Primary kidney cell culture morphology from (**a**) rat, (**b**) canine, (**c**) human kidney prior to harvest and passaging (×100)

10. Remove culture medium by pouring into a waste bottle (ensuring to maintain sterile technique) (see Note 8).
11. Wash each culture vessels with 25 ml sterile PBS (for T500). Add enough PBS to remove all traces of culture media (especially serum) and cellular debris. Volume will vary depending on the culture vessel.
12. Remove PBS wash via pouring into a waste bottle.
13. Add appropriate volume of pre-warmed trypsin to cell monolayer at a volume that is recommended by culture vessel manufacturer (for a T500 add 25 ml of trypsin).
14. Monitor cell layer via light microscopy until cells have detached. This should take typically 5–7 min but less than 10 min.
15. Prepare a sterile collection vessel for collection of the trypsinized cells. Add a volume of 5% FBS DMEM or other comparable medium to the vessel such that the neutralization medium is equivalent to 10–20% of the final collected volume (see Note 9).
16. When the cells have detached, gently tap flasks to detach all cells. Pour the trypsinized contents into the collection vessel.
17. Continue process until all of the cells have been harvested. Stagger process if possible so that cells are not exposed to trypsin for extended times.
18. Transfer neutralized cell suspension to conical tubes for centrifugation ($300 \times g$ for 5 min).
19. Aspirate supernatant and after tapping to loosen pellet, resuspend in KSFM in sufficient volume for counting. Variation of buffers used in the wash/prep process can affect the banding of cells on the density gradient and should be avoided.
20. Remove 18 μl cell sample for counting (using either a hemacytometer or an automatic cell counter) (see Note 10).
21. Record live cell, dead cell, cell viability, cells per ml, and total number of cells on batch record. Prepare sample of unfractionated (pre-gradient) cells for analysis.

22. Determine the distribution of cells that will be fractionated or banded for injection/analysis, cryopreserved, designated for flow cytometry, WB, or other functional/characterization-based needs.
23. If applicable, aliquot the portion of cells that are to be cryopreserved into 50 ml conical tube(s) previously described in Subheading 3.2.
24. Aliquot the portion of cells that are to be fractionated/banded into 50 ml conical tube(s).
25. Centrifuge all tubes at 300 × *g* for 5 min if using 50 ml conical tubes.
26. Carefully aspirate supernatant via vacuum aspiration and discard, leaving only cell pellet at the bottom of the tube.
27. Tap tube to loosen pellet and resuspend cells.
28. Resuspend pellet such that the concentration of cells is equivalent to 30–37.5e6 cells per ml. These cells will be used for subfractionation using a discontinuous Optiprep step gradient in the next section.
29. At this point the cells are ready to be fractionated/banded and cryopreserved for later culture.

3.4 Renal Cell Fractionation

1. Determine the amount of subcultured primary cells that have been attained.
2. Each density step gradient requires a cell number ranging from 60 to 75 million cells per tube. Once the number of cells has been determined, calculate the number of gradient tubes to be generated.
3. Prepare 7 and 16% Optiprep as previously described in sufficient volumes to prepare needed gradients. Typically no less than 40 ml is prepared to minimize pipetting error associated with smaller volumes.
4. Make a discontinuous density step gradient(s) by first pipetting 4 ml of 16% Optiprep in KSFM into each of the required 15 ml conical centrifuge tube(s) to form the bottom layer of the two-step gradient.
5. Carefully layer 4 ml of 7% Optiprep in KSFM onto the bottom layer of the density gradient by tilting the tube at a 45° angle and letting the medium slowly run down the side of the tube. This will minimize mixing at the interface between the two different densities.
6. Once the top of the two-step density gradient has been layered, carefully pipette 2 ml of cell suspension containing between 60 and 75 million cells in KSFM medium on top of the step gradient using the above layering method. Continue until all of the gradients have been loaded with cell suspension.

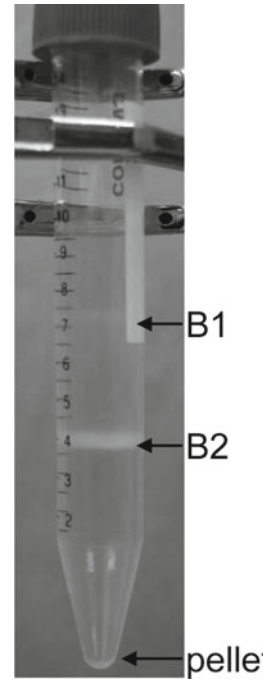

Fig. 2 Discontinuous OptiPrep 2—step gradient. 7% (w/v) layered on top of the 16% (w/v) provide an interface for the biologically active cells to settle. The upper Band 1 (*B1*) cells consist of the larger tubular epithelial cells as well as collecting duct cells while the Band 2 (*B2*) cells comprise a majority of proximal tubular cells. The pellet composition is mainly dead cell and debris

7. Once the cells have been loaded, carefully place tubes into the centrifuge and spin at 800 × *g* for 20 min without brake.
8. After centrifugation, collect tubes and visually inspect gradient bands to verify banding pattern. Between the 7–8 ml mark there should be a thin band of cells referred to as Band 1. A heavy second band should be visible near the 4 ml mark and is referred to as Band 2. A faint pellet of cells will also be present (Fig. 2).
9. Collect gradient bands by aspirating each band using either sterile bulb or 5 ml pipette.
10. Place each cell band in a separate tube. Separately combine all Band 1's, Band 2's, and then pellets.
11. Once all bands have been collected and combined, dilute at least twofold with KSFM medium and mix well by inversion. Resuspend the residual pellet(s) with equal volume of KSFM.
12. Wash out residual Optiprep by centrifuging at 300 × *g* for 5 min.
13. Resuspend the pellets from all of the collected bands in 50:50 medium and perform cell count.

14. After cell count has been determined, collect samples for cell analysis or cryopreserve the remaining cells using previously described methods for later use.
15. The biologically active Band 2 cells from rodent and canine have been successfully used to treat model chronic kidney disease.

4 Notes

1. Example: Stock collagenase = 17,440U. 17,440U/300U = 58 ml of dispase (at 4 U/ml) needed to add to collagenase to make collagenase 300 U final.
2. Initial unfractionated cells are very "tube-like" in appearance and it can be difficult to count individual cells.
3. Example of trypan blue exclusion using hemocytometer: To make a count using a 1.1× dilution, take 18 µl cell sample and 2 µl of 0.4% trypan blue, load 10 µl of cell count mixture, and view through upright light microscope. Count at least two squares of the grid and calculate the number of viable cells for instance:
   (a) For a 1.1 (dilution factor) × 10,000 × (no. of live cells/no. of squares counted) = total live cells/ml.
   (b) Multiply total live cells/ml by total volume of neutralized collection volume = total no. of live cells.
4. Testing initial unfractionated cells will provide information on baseline expression levels for RNA (or Western Blot) as described below. Samples can be taken at this point for additional testing as needed for FACS analysis using Guava or for functional analysis such as GGT/LAP. Make sure that all samples are correctly labeled.
   (a) Remove 1e6 cells for RNA isolation (and 1e6 for Western Blot if applicable) and place in a sterile 1.5 ml microcentrifuge tube.
   (b) Add DBPS to microcentrifuge tube(s) (for wash step) and centrifuge at 300 × *g* for 5 min. Aspirate contents down to pellet and snap freeze pellet in liquid nitrogen. (Label tube with assigned RNA number from RNA folder indicating sample designation and record in batch record.)
   (c) Cells taken for flow analysis are washed in DPBS, fixed in 2% paraformaldehyde for 30 min, then washed, and stored at 4°C.
5. It is possible to load 225e6 cells in 50 ml conical tubes.
6. If too little KSFM is added and/or if tubes are not mixed well, the Optiprep mixture will re-band the cells.

7. Recommended seeding for T500 Nunc flask: 25e6 cells/flask, 150 ml of 50:50 media. This sample will be passage 0 (p0) once seeded. If cultures will be harvested 4 days later, plate at 30,000 cells/cm^2. It is recommended to vent flask caps, even if flasks have filter caps, to promote adequate gas exchange across cell monolayer.
8. Contamination mat arises if the collection procedure was not performed using aseptic technique. If contamination is observed, immediately remove all culture vessels from incubator. Save contaminated medium for microbiological analysis. Dispose of contaminated material properly with bleach and thoroughly clean BSC and incubators with Bacdown and 70% EtOH (or autoclave contaminated materials if applicable).
9. If 10×T500 flasks are to be trypsinized, the total trypsinized volume will be 250 ml. Add 25–50 ml of neutralization buffer to the collection vessel.
10. The harvested cultured kidney cells are usually in a fairly uniform single-cell suspension, so it is possible for an automated counter to get an accurate count at this point such as a Cedex or an equivalent.

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