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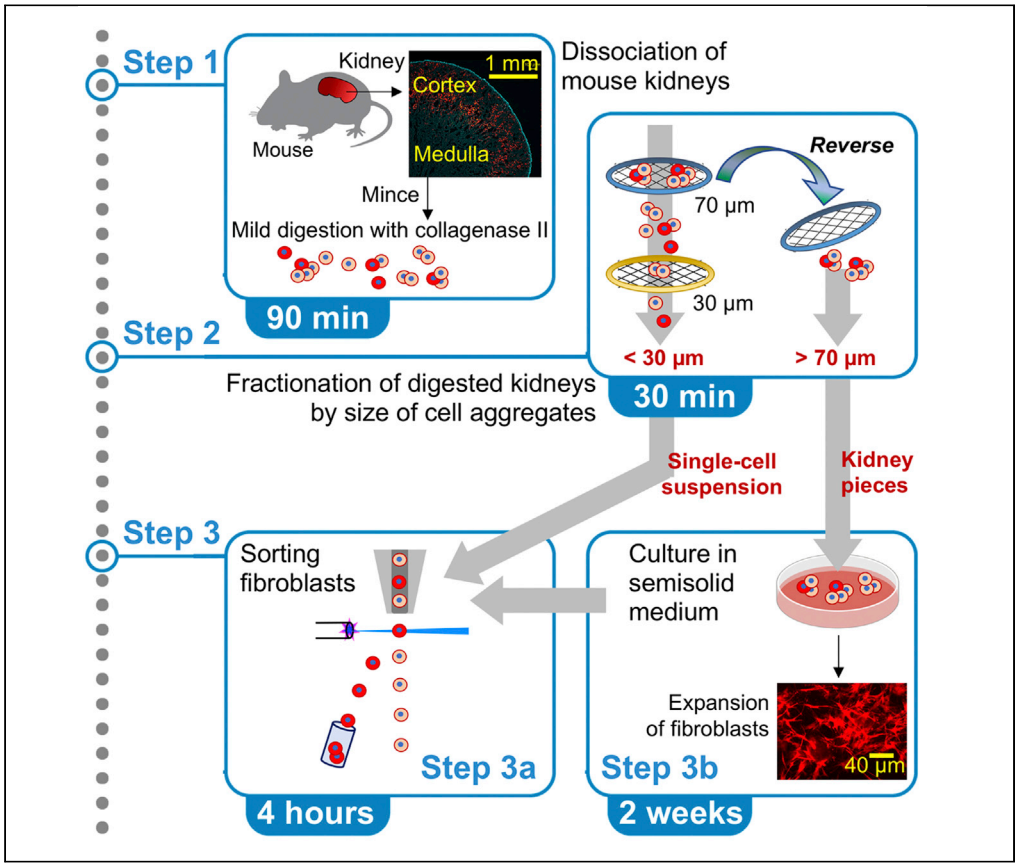
Protocol

Efficient isolation of interstitial fibroblasts directly from mouse kidneys or indirectly after *ex vivo* expansion



Renal interstitial fibroblasts are responsible for producing the erythroid growth factor Epo and the vasopressor renin in addition to kidney fibrosis, in which they are transformed into myofibroblasts. Therefore, analyses of fibroblasts may elucidate the complex mechanisms of kidney diseases. However, the fragility of these cells makes their isolation for *in vitro* analyses and *ex vivo* cultivation difficult. We have overcome these difficulties by mildly dissociating mouse kidneys and coculturing fibroblasts with other kidney cells in semisolid medium.

Taku Nakai, Yuma Iwamura, Norio Suzuki

sunorio@med.tohoku.ac.jp

Highlights

A cell sorter-based protocol for isolation of renal interstitial fibroblasts from mice

A protocol for *ex vivo* expansion of interstitial fibroblasts from kidney pieces

Cells isolated with this protocol are available for culture and single-cell analyses

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Efficient isolation of interstitial fibroblasts directly from mouse kidneys or indirectly after *ex vivo* expansion

Taku Nakai,[1] Yuma Iwamura,[1] and Norio Suzuki[1,2,3,*]

[1]Division of Oxygen Biology, United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai, Miyagi 980-8575, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: sunorio@med.tohoku.ac.jp
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SUMMARY

Renal interstitial fibroblasts are responsible for producing the erythroid growth factor Epo and the vasopressor renin in addition to kidney fibrosis, in which they are transformed into myofibroblasts. Therefore, analyses of fibroblasts may elucidate the complex mechanisms of kidney diseases. However, the fragility of these cells makes their isolation for *in vitro* analyses and *ex vivo* cultivation difficult. We have overcome these difficulties by mildly dissociating mouse kidneys and coculturing fibroblasts with other kidney cells in semisolid medium.
For complete details on the use and execution of this protocol, please refer to Sato et al. (2019a) and Miyauchi et al. (2021).

BEFORE YOU BEGIN

This protocol describes how to efficiently isolate living interstitial fibroblasts from mouse kidneys and expand them in *ex vivo* cultivation. Because a subset of interstitial fibroblasts in the renal cortex, which are referred to as renal erythropoietin (Epo)-producing (REP) cells, play key roles in Epo production, renin production and kidney fibrosis (Obara et al., 2008; Souma et al., 2013, 2016; Suzuki and Yamamoto, 2016; Miyauchi et al., 2021), this protocol features the manipulation of REP cells. This protocol was established using a genetically modified mouse line in which REP cells were efficiently labeled with the tdTomato red fluorescent protein *via* the transgene expression of Cre recombinase under the control of mouse *Epo* gene regulatory regions (Yamazaki et al., 2013; Suzuki et al., 2016). All animal experiments reported herein were approved by the Animal Care Committee of Tohoku University (approval number 2020-MdA-078).

Based on our experience, primary REP cells can hardly survive or expand when cultured *ex vivo* without other kidney cells, such as tubular epithelial cells and capillary endothelial cells (Sato et al., 2019a). Therefore, two alternative methods for REP cell isolation, direct isolation from mouse kidneys and isolation after kidney cell coculture, are introduced below (Figure 1).

Preparation of reagents

Timing: 20 min

1. Prepare the required volume of Collagenase II solution (5 mL per kidney).
2. Cool the Collagenase II solution and PBS to 4°C.
3. Warm the required amount of DMEM supplemented with or without 10% fetal bovine serum (FBS) to 37°C (30 mL for each is enough for the whole protocol for a kidney).
4. Warm a shaking incubator to 37°C.

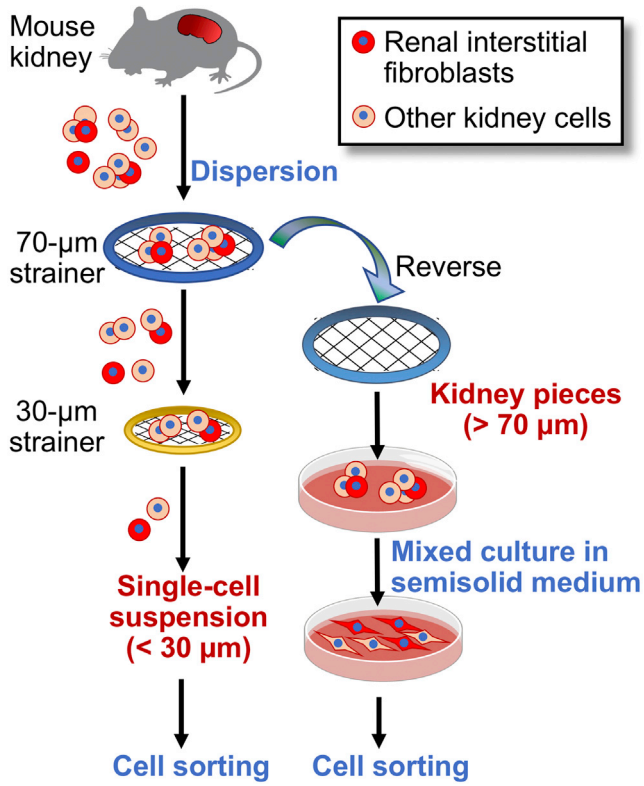

Figure 1. Overview of the protocol to isolate interstitial fibroblasts from a mouse kidney

A mouse kidney is minced and digested with Collagenase II before being serially filtered with 70-μm and 30-μm cell strainers. The flow-through (single-cell suspension) from the 30-μm cell strainer is directly utilized for cell sorting to isolate primary renal interstitial fibroblasts. Remnants (kidney pieces) on the 70-μm strainer are collected and cultivated in semisolid medium for 1–2 weeks to expand renal interstitial fibroblasts, followed by cell sorting.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mouse kidneys (C57BL/6J, 8–15 weeks of age)	Tohoku University	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle medium (DMEM), high glucose	Nacalai Tesque	Cat# 08459-35
FBS	Biosera	Cat# FB-1003/500 Batch# 018BS514
Phosphate-buffered saline (PBS) (pH 7.4, 10×)	Nacalai Tesque	Cat# 27575-31
Collagenase II	Worthington Biochemical	Cat# CLS-2
MethoCult M3231	STEMCELL Technologies	Cat# 03231
Accutase in DPBS without Ca, Mg	Nacalai Tesque	Cat# 12679-54
Antibiotic-antimycotic mixed stock solution (100×)	Nacalai Tesque	Cat# 09366-44
Software and algorithms		
BD FACS software	BD Biosciences	N/A
Other		
BD FACSJazz	BD Biosciences	N/A
gentleMACS C tubes	Miltenyi Biotec	Cat# 130-093-237

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
gentleMACS Dissociator	Miltenyi Biotec	Cat# 130-093-235
EASYstrainer for 50-mL tube, 70-µm mesh size	Greiner Bio-One	Cat# 542070
Preseparation filter (30 µm)	Miltenyi Biotec	Cat# 130-041-407

MATERIALS AND EQUIPMENT

Collagenase II solution (for digestion of a mouse kidney)		
Reagent	Final concentration	Amount
DMEM, high glucose	-	5 mL
Collagenase II	1 mg/mL	5 mg
Total	-	5 mL
Prepare immediately before use.		

Semisolid culture medium		
Reagent	Final concentration	Amount
DMEM, high glucose	45%	9 mL
FBS, heat-inactivated	5%	1 mL
MethoCult M3231	50%	10 mL
Total	-	20 mL

STEP-BY-STEP METHOD DETAILS

Dissociation of a mouse kidney

Timing: 2 h

This step demonstrates how to prepare a cell suspension from a mouse kidney for the isolation and cultivation of renal interstitial fibroblasts. Reagent volumes are determined by multiplication of the number of mouse kidneys being prepared. Renal interstitial fibroblasts tightly weave around capillaries in the kidney (Souma et al., 2016) and are likely vulnerable to physical stress. Therefore, to maintain the intrinsic features of isolated fibroblasts, mouse kidneys should be finely dissociated as gently as possible.

1. Harvest a mouse kidney and remove the fibrous capsule and the renal medulla using forceps (Figure 2A).
2. Mince a kidney with scissors on a Petri dish at room temperature (20°C–25°C; Figure 2B).
3. Add 5 mL of ice-cold PBS and transfer the minced kidney into a C-tube.
4. Remove the supernatant (PBS) after 1 min of standing on ice (Figure 2C).
5. Gently mix the minced kidney with 5 mL of ice-cold PBS by pipetting.
6. Repeat steps 4 and 5 three times.
7. Remove the supernatant (PBS) after 1 min of standing on ice (Figure 2C).
8. Resuspend the minced kidney with 5 mL of the prewarmed Collagenase II solution (see materials and equipment).

⚠ CRITICAL: For a better yield, the kidneys should be minced well (Figure 2B) and incubated with freshly prepared Collagenase II solution.

9. Incubate the cell suspension for 1 h at 37°C in a prewarmed shaking incubator (130 rpm).

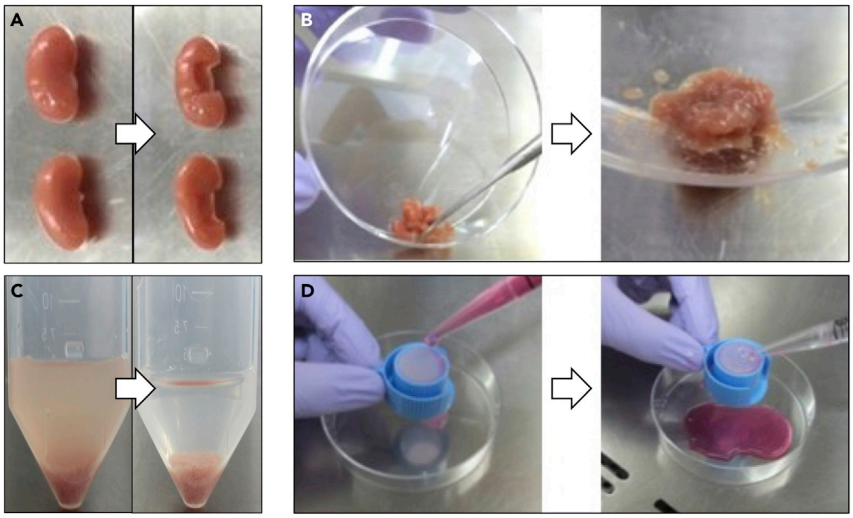

Figure 2. Preparation of kidney pieces

(A) Mouse kidneys before (left) and after (right) renal medulla removal.

(B) Kidneys are minced with scissors (left) until they become a smooth paste (right).

(C) Minced kidneys are washed with PBS in a C-tube three times. The left and right panels show the first and third washes, respectively. Clear supernatant (PBS) is removed after 1 min of standing.

(D) Kidney pieces are collected on a 70-μm strainer. The remnants on the 70-μm strainer are collected by washing the strainer, which is face down, with DMEM containing FBS.

10. Stir the cell suspension in a C-tube with a gentleMACS Dissociator (Program B: 553 rpm for 30 s) two times.
11. Centrifuge (300×*g*) the mixture for 5 min at 4°C and remove the supernatant.
12. Resuspend the cell pellet in 5 mL of DMEM containing FBS (10%, v/v).
13. Filter the cell suspension through a 70-μm cell strainer in a 50-mL tube.
    a. Flow-through: for step 14 (isolation of primary renal interstitial fibroblasts)
    b. Remnants: for step 20 (cultivation of kidney pieces)

Isolation of primary renal interstitial fibroblasts

Timing: >2 h

14. (From step 13a) Filter the cell suspension through a Preseparation filter (a 30-μm cell strainer) in a 15-mL tube.
15. Measure the cell concentration of the flow-through fraction to determine the volume of single-cell suspensions at step 17.
16. Centrifuge (300×*g*) the mixture for 5 min at 4°C and remove the supernatant.
17. Resuspend the cells in DMEM containing FBS (10%, v/v) at a concentration of approximately 1×10^6 cells/mL and store on ice.
18. To stain the cells with antibodies that are conjugated with fluorescent dye appropriate for flow cytometry, incubate the cell suspension with antibodies (1:100 dilution) for 40 min on ice, followed by washing the antibodies 3 times with DMEM containing FBS (10%, v/v).

Note: To specifically stain renal interstitial fibroblasts of mouse kidneys, antibodies against CD73 (BioLegend Cat# 127207) and PDGFRβ (Abcam Cat# ab91066) are often used (Pan et al., 2011; Higashi et al., 2019; Conway et al., 2020; Geng et al., 2021).

Alternatives: Using genetically modified mice that express fluorescent proteins exclusively in interstitial fibroblasts in kidneys, antibody staining can be omitted. Indeed, this protocol was

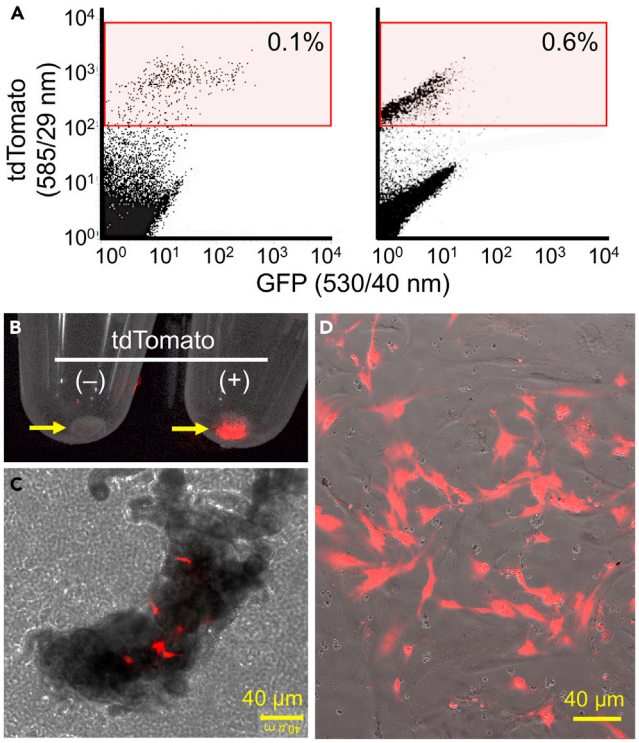

Figure 3. Isolation of REP cells from REP cell-reporter mice before or after cultivation in semisolid medium

(A) The expression of tdTomato red fluorescent protein and green fluorescent protein (GFP) in kidney cell suspensions of anemic REP cell-reporter mice before (left, step 19) and after (right, step 37) primary culture for 6 days is analyzed by flow cytometry. In the reporter mice, tdTomato is expressed by Cre-mediated recombination of the *Rosa26-loxP-STOP-loxp-tdTomato* modified gene in cells that have ever expressed the *EpoCre* transgene (Yamazaki et al., 2013). The anemic condition of the reporter mouse enhances the expression of GFP from the endogenous *Epo* allele (left), in which GFP cDNA is integrated, because *Epo* gene expression is induced by anemic hypoxia (Obara et al., 2008). During the primary culture, GFP expression disappears (right). The percentage of tdTomato$^+$ cells (red boxes) increases from 0.1% to 0.6% after primary culture.

(B) tdTomato fluorescence is detectable in cell pellets (arrows) of sorted tdTomato$^+$ fibroblasts (right) but not in those of tdTomato$^-$ cells (left).

(C) tdTomato expression in a kidney piece from the REP cell-reporter mouse.

(D) tdTomato expression in cells primarily cultured for 6 days in semisolid medium.

optimized by using a genetically modified mouse line expressing tdTomato fluorescence in REP cells, which is referred to as the "REP cell-reporter mouse line" in this protocol (Yamazaki et al., 2013).

19. Sort fibroblasts with a cell sorter (Figure 3A). The settings of a FACSJazz cell sorter are as below.

Nozzle size: 100 μm

Sort mode: 1.5 Drop Pure

Event Rate: 1000–2000 cells/s

Note: Using this protocol, it usually takes approximately 3 h to collect 3000 REP cells from the kidneys of a REP-cell reporter mouse, in which approximately 0.1% of total cells are tdTomato-positive REP cells (see Figure 3A). The viability of sorted cells is not decreased until at least 3 h on ice.

Cultivation of kidney pieces

Timing: 7–14 days

20. (From step 13b) Flip the 70-μm cell strainer over onto a dish to collect the remnant cells on the strainer (Figure 2D).
21. Flush the cell strainer with 6 mL of DMEM containing FBS (Figure 2D).
22. Centrifuge (300×*g*) the mixture for 5 min at 4°C and remove the supernatant.
23. Resuspend the kidney pieces (Figure 3C) in 6 mL of the semisolid culture medium (see materials and equipment).
24. Seed the kidney pieces on dishes or plates.
25. Incubate the cells for 7–14 days at 37°C in a CO_2 (5%) incubator (Figure 3D). The medium does not need to be changed.
26. Harvest the cells by pipetting 10 times the PBS volume and place the cells in a 50-mL tube.
27. Centrifuge (300×*g*) the mixture for 5 min at 4°C and discard the supernatant.
28. Resuspend the cell pellet in 10 mL of PBS.
29. Centrifuge (300×*g*) the mixture for 5 min at 4°C and discard the supernatant.
30. Resuspend the cell pellet in 500 μL of Accutase and incubate the mixture at 37°C for 10 min.
31. Add 3 mL of DMEM containing FBS.
32. Filter the cell suspension through a Preseparation filter (a 30-μm cell strainer) in a 15-mL tube.
33. Determine the cell concentration in the flow-through fraction.
34. Centrifuge (300×*g*) the mixture for 5 min at 4°C and remove the supernatant.
35. Resuspend the cells in DMEM containing FBS at a concentration of approximately 1×10^6 cells/mL and store on ice.
36. Stain the cells with antibodies and sort the cells according to steps 18 and 19 (Figure 3A).

EXPECTED OUTCOMES

Following this protocol, we collected approximately 3000 primary REP cells on average from the kidneys of an adult REP cell-labeled mouse (Yamazaki et al., 2013). Sufficient amounts (1×10^5 cells) of cells for reverse transcription-quantitative PCR (RT-qPCR) analyses were collected by repeating steps 1–19 of this protocol (Souma et al., 2013; Miyauchi et al., 2021). Alternatively, to conduct RT-qPCR analyses with a small number of REP cells, a CellAmp Whole Transcriptome Amplification Kit (Takara, Cat# 3734) was successfully used. Additionally, cells isolated with this protocol are available for single-cell RNA sequencing.

Isolated primary REP cells died within one week under all of the *ex vivo* conditions that we tested. For long-term cultivation of renal interstitial fibroblasts, the cells likely require acclimation to *ex vivo* conditions by incubation with other kidney cells for more than one week, as described in steps 20–25 of this protocol (Sato et al., 2019a). During kidney cell coculture, it is possible to analyze cellular responses to chemical, physical, and biological stimuli. Since renal interstitial fibroblasts are reported to contain mesenchymal stem cells (Plotkin and Goligorsky, 2006; Bussolati et al., 2013), their multilineage differentiation potential can be investigated by the semisolid medium coculture.

This protocol for the isolation of fibroblasts from healthy kidneys is applicable to the isolation of myofibroblasts from fibrotic kidneys, which originate from REP cells in injured kidneys (Souma et al., 2013; Sato et al., 2019a, 2019b). Because myofibroblasts occupy a large portion of fibrotic kidneys in mice subjected to unilateral ureteral obstruction, sufficient numbers of myofibroblasts are isolated from the mice for molecular and cellular analyses (Souma et al., 2013). The mechanisms of kidney fibrosis, a common pathway to kidney diseases, should be elucidated by comparing isolated fibroblasts with isolated myofibroblasts. In contrast to fibroblasts, isolated primary myofibroblasts grow well under *ex vivo* conditions (Chang et al., 2016).

LIMITATIONS

To label a subpopulation of renal interstitial fibroblasts, we usually use genetically modified mouse lines, in which transgenic Cre recombinase or fluorescent proteins are specifically expressed in REP cells under the control of the *Epo* gene regulatory region (Yamazaki et al., 2013, 2021; Suzuki et al., 2013, 2016; Suzuki and Yamamoto, 2016; Hirano et al., 2017). In addition to the *Epo* gene regulatory regions, various genes expressed in renal interstitial fibroblasts are used to express fluorescent proteins in the cells of genetically modified mice (Chang et al., 2016; Kramann et al., 2015; Kuppe et al., 2021). Although these reporter mouse lines allow the identification of REP cells or renal fibroblasts in culture dishes (Figure 3C and 3D) and the sorting of cells without antibody staining, they are inappropriate for general use and for human pathology applications. Instead of reporter mice, antibodies targeting cell surface antigens are available for specifically labeling interstitial fibroblasts in kidney cell suspensions. Our previous flow cytometry analysis demonstrated that an anti-CD73 antibody (BioLegend Cat# 127207) recognized fibroblasts, including REP cells, in neonatal mouse kidneys (Pan et al., 2011). Renal interstitial fibroblasts can also be specifically stained with an anti-PDGFRβ antibody (Higashi et al., 2019; Broeker et al., 2020; Conway et al., 2020; Geng et al., 2021).

Isolated renal fibroblasts are easily transformed into myofibroblasts under *in vitro* conditions, thereby inducing proliferation (Sato et al., 2019a). This observation suggests that the *in vivo* microenvironment of healthy kidneys is essential for maintaining the intrinsic features of interstitial fibroblasts because they are transformed into vigorously proliferating myofibroblasts in injured kidneys. Additionally, physical stress from cell sorting may stimulate transformation (Beliakova-Bethell et al., 2014).

TROUBLESHOOTING

Problem 1

Very few renal interstitial fibroblasts are detected by flow cytometry at step 19.

Potential solution

The kidneys should be thoroughly minced (step 3, Figure 2C). Additionally, minced kidneys should be incubated with Collagenase II solution, which must be prepared just before use, for more than 1 h (step 9). When myofibroblasts are isolated from fibrotic mouse kidneys (Souma et al., 2013), the minced kidneys are recommended to be digested with Collagenase II solution for 2 h.

Problem 2

The fibroblast purity is very low after cell sorting.

Potential solution

Flow cytometry-based sorters fundamentally isolate cells as single cells in each droplet. However, we found that REP cells and renal interstitial fibroblasts are often isolated with other cells as doublets. This problem is thought to be caused by the mild dissociation of kidneys (steps 1–19) to maintain their intrinsic ability to tightly adhere to endothelial cells (Souma et al., 2016). Since extensive kidney dissociation can result in renal interstitial fibroblast injury, cell sorters should be used in the "single drop mode" to prohibit droplets carrying more than 2 cells from being obtained and thereby securely prevent the contamination of cells other than the targeted fibroblasts at the expense of the yield.

Problem 3

Flow cytometry detects renal interstitial fibroblasts positive for antibody (anti-CD73 or PDGFRβ antibodies) staining at low frequency in a kidney cell suspension (step 19).

Potential solution

In the case that the frequency is lower than the normal range (approximately 0.1%), the staining strategy can be replaced with a negative selection, which uses anti-CD45 (hematopoietic cells; BD

Biosciences Cat# 553081), anti-CD326 (tubular epithelial cells; Biologend Cat# 118208), and anti-CD31 antibodies (vascular endothelial cells; eBioscience Cat# 12-0311-82).

Problem 4

The majority of cells do not adhere to the dish bottom after seeding kidney pieces (step 24).

Potential solution

Renal fibroblasts should creep out and migrate from kidney pieces within the first week of the mixed culture (step 24), and adhere to the bottom of polystyrene dishes/plates for their growth (Figures 3C and 3D). To improve the adherent efficiency, concentrations of MethoCult in semisolid culture medium could be altered to 40% or 60% instead of the 50% that we recommend in this protocol.

Problem 5

Cells isolated with this protocol (step 24 or step 37) hardly survive in culture dishes/plates.

Potential solution

The optimum conditions for culturing isolated renal interstitial cells have not yet been established. The cells are considered to require other renal cells for their *ex vivo* growth, such as cultivation of kidney pieces, which is introduced in this protocol. We have experienced that the semisolid medium used in this protocol for cultivation of kidney pieces is superior to normal liquid medium for the cultivation of isolated renal interstitial cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Suzuki Norio, Ph.D. (sunorio@med.tohoku.ac.jp).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This protocol did not analyze datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T.N., Y.I., and N.S. developed the protocol. T.N. and N.S. prepared the figures and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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