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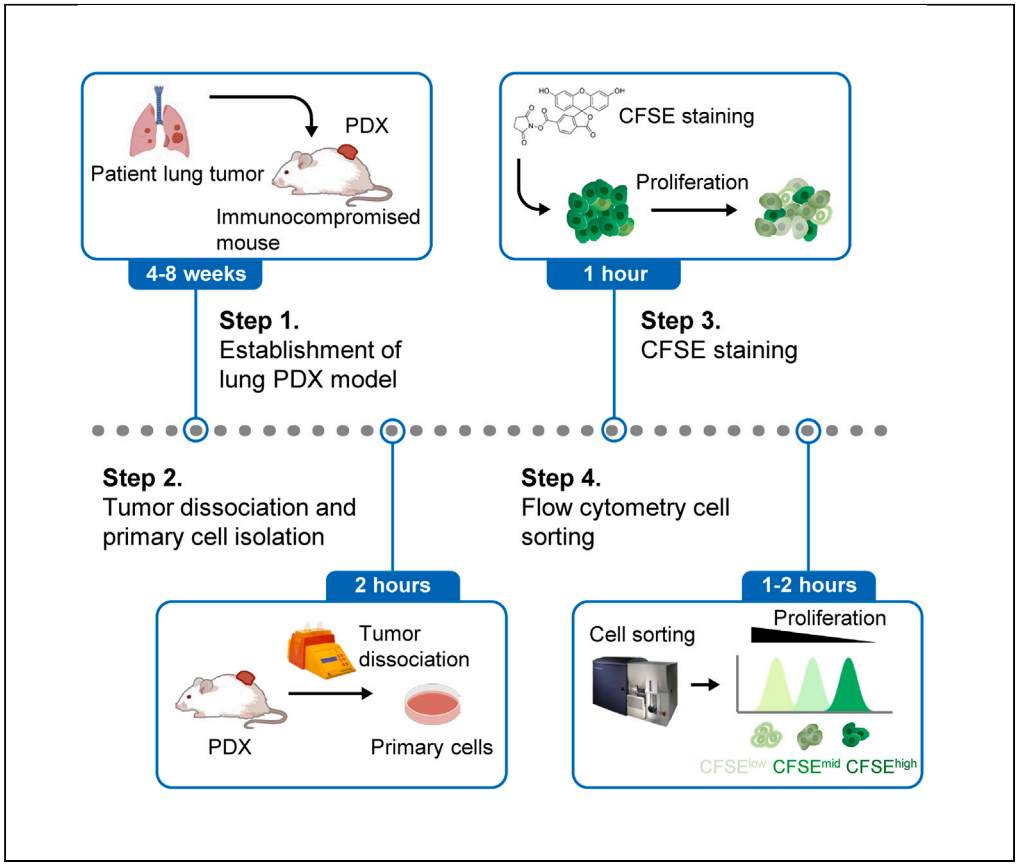


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## Highlights

Reliable establishment of lung PDX mouse model

Efficient dissociation of PDX tumor and construction of PDX-derived primary cells

Isolation of slow-cycling cancer cells based on the level of CFSE dye retention

Proliferation level comparison among the $CFSE^{low}$, $CFSE^{mid}$, and $CFSE^{high}$ populations

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# Isolation of slow-cycling cancer cells from lung patient-derived xenograft using carboxyfluorescein-succinimidyl ester retention-mediated cell sorting

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## SUMMARY

**The slow-cycling subpopulation plays an important role in anticancer drug resistance and tumor recurrence. Here, we describe a clinically relevant patient-derived xenograft model and a carboxyfluorescein succinimidyl ester dye that is diluted in a cell proliferation-dependent manner. We detail steps to separate active-cycling cancer cells and slow-cycling cancer cells (SCCs) in heterogeneous cancer populations to confirm their different cellular properties. This protocol can be used to distinguish SCCs, investigate their biology, and develop strategies for anticancer therapeutics.**

**For complete details on the use and execution of this protocol, please refer to Cho et al. (2021).[1]**

## BEFORE YOU BEGIN

This protocol describes the process of lung patient-derived xenograft (PDX) model establishment and dissociation of the tumor from *in vivo* mouse model followed by the separation of cell populations depending on their proliferation rate. In this protocol we showed how to establish viable lung PDX primary cells and isolate active-cycling cancer cells (ACCs) and slow-cycling cancer cells (SCCs) from lung PDX-derived primary cells by using the carboxyfluorescein succinimidyl ester (CFSE) dye. CFSE staining has been applied to several immune cells for analyzing their proliferation rate.[2,3] The other cell staining dyes that are diluted by cell division, for example, PKH26 which incorporates cell plasma membrane, can substitute CFSE for the same purpose.

This protocol can be applied not only to lung PDX primary cells but also colon PDX primary cells and established lung cancer cells. Researchers are recommended to optimize the CFSE staining conditions (concentration and incubation time) depending on their experimental settings.

### Institutional permissions

Before performing this protocol, researchers should ensure that any human-relevant clinical specimens and *in vivo* experiments comply with their institutional guidelines. In this study, all animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (approval no. SNU-170228-1).

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological samples | | |
| Lung patient-derived xenografts (PDX) | Cho et al.[1] (Ref. #3) | N/A |
| Experimental models: Organisms/strains | | |
| 6–8-week-old female NOD.Cg-Prkdcscid/J (NOD/SCID) | Jackson Laboratory | Strain #001303 RRID: IMSR_JAX:001303 |
| Antibodies | | |
| CD45-APC (1:200 dilution) | BioLegend | 157605 |
| CD31-APC (1:200 dilution) | BioLegend | 102409 |
| PDGFRα-APC (1:200 dilution) | BioLegend | 135907 |
| Chemicals, peptides, and recombinant proteins | | |
| Matrigel® Basement Membrane Matrix | Corning | 354234 |
| Ketamine | Yuhan | N/A |
| Xylazine hydrochloride (Rompun®) | BAYER | N/A |
| Dulbecco's phosphate buffered saline (DPBS) | Welgene | LB 001-02 |
| RPMI 1640 | Welgene | LM 011-01 |
| Fetal bovine serum | Welgene | S 101-07 |
| Penicillin-streptomycin solution (100×) | Welgene | LS 202-02 |
| Collagenase D | Roche | 11088858001 |
| DNaseI | Sigma | D4527 |
| Trypsin-EDTA solution (10×) | Welgene | LS 015-02 |
| Bovine serum albumin (BSA) | GenDEPOT | A0100-100 |
| Ethylenediaminetetraacetic acid (EDTA) | Amresco | 0105 |
| Crystal violet | Sigma | C0775 |
| Methanol | Sigma | 494437 |
| Critical commercial assays | | |
| Tumor Dissociation Kit human | Miltenyi Biotec | 130-095-929 |
| gentleMACS C Tubes | Miltenyi Biotec | 130-093-237 |
| gentleMACS Dissociator | Miltenyi Biotec | 130-093-235 |
| CFSE Cell Division Tracker Kit | BioLegend | 423801 |
| Software and algorithms | | |
| FACSDiva software | BD Biosciences | N/A |
| FlowJo version 10.3 | BD Biosciences | N/A |
| Other | | |
| 60 mm culture dish | SPL | 11060 |
| 90 mm culture dish | SPL | 11090 |
| 12 well culture plate | SPL | 30012 |
| Nunc™ Biobanking and Cell Culture Cryogenic Tubes | Thermo Scientific | 368632 |
| Disposable scalpel | Feather | 0210 #10 |
| Stainless steel trocar needle | Jeung-Do Bio & Plant | JD-S-98 |
| Sterile suture with C3 needle | AILEE | SK439 |
| 1 mL insulin syringe | BD Biosciences | 320320 |
| Cell strainer 70 μm | SPL | 93070 |
| Trypan blue solution, 0.4% | Gibco | 15250061 |
| Falcon® 5 mL round bottom polystyrene test tube, with cell strainer snap cap | Corning | 352235 |
| 15 mL conical tube | SPL | 50015 |
| 1.5 mL microcentrifuge tube | Hyundai Micro | H23015 |
| Syringe filter (0.2 μm) | Corning | 431219 |
| FACS Aria III cell sorter | BD Biosciences | N/A |

## MATERIALS AND EQUIPMENT

**Anesthesia cocktail**

| Reagent | Final concentration | Amount |
|---|---|---|
| Ketamine (50 mg/mL) | 20 mg/mL | 400 μL |
| Xylazine hydrochloride (Rompun®) (20 mg/mL) | 1 mg/mL | 50 μL |
| Dulbecco's phosphate buffered saline (DPBS) | N/A | 550 μL |
| Total | | 1 mL |

*Prepare the solution in a sterile condition. Store at 4°C for up to 2 weeks.

**Complete cell culture media (RPMI 1640)**

| Reagent | Final concentration | Amount |
|---|---|---|
| RPMI 1640 | N/A | 500 mL |
| Fetal bovine serum | 10% | 50 mL |
| Penicillin-streptomycin solutions (100×) | 1% (100 U/mL penicillin and 100 μg/mL streptomycin) | 5.55 mL |
| Total | | 555.6 mL |

*Prepare the buffer in a sterile condition. Store at 4°C for up to 4 weeks.

Trypsin-EDTA Solution (2×)

| Reagent | Final concentration | Amount |
|---|---|---|
| Trypsin-EDTA Solution (10×) | 2× | 10 mL |
| Dulbecco's phosphate buffered saline (DPBS) | N/A | 40 mL |
| Total | | 50 mL |

*Prepare the solution in a sterile condition. Store at 4°C for up to 4 weeks.

**Flow cytometry buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| Bovine serum albumin (BSA) | 0.5% | 0.25 g |
| Ethylenediaminetetraacetic acid (EDTA) | 2 mM | 37.22 mg |
| Dulbecco's phosphate buffered saline (DPBS) | N/A | 50 mL |
| Total | | 50 mL |

*Prepare the buffer in a sterile condition. Store at 4°C for up to 4 weeks.

**CFSE staining dye**

| Reagent | Final concentration | Amount |
|---|---|---|
| Lyophilized 100 μg CFSE | 5 mM | 100 μg |
| Anhydrous DMSO | N/A | 36 μL |
| Total | | 36 μL |

*Prepare the solution in a sterile condition. Protect from light. Store at −20°C for up to 4 weeks.

**Crystal violet solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| Crystal Violet | 0.5% | 0.5 g |
| Distilled $H_2O$ | N/A | 80 mL |
| Methanol | 20% | 20 mL |
| Total | | 100 mL |

*Protect from light. Store at room (22 ± 2°C) temperature (RT) for up to 2 months.

## STEP-BY-STEP METHOD DETAILS

### Establishment of lung PDX mouse model

Timing: 4–8 weeks

In this section, we describe the establishment of the lung PDX model with NOD/SCID mice.

*Note:* Stock of PDX sample should be stored in a liquid nitrogen tank with screw cap cryogenic tube. Do not repeat the freeze-thaw cycle to keep the viability of the sample.

*Note:* Unless specified instructions, it is strongly recommended to perform the process below in sterile conditions to prevent possible contamination.

1. Take a frozen lung PDX sample out from a liquid nitrogen storage tank.

*Note:* There is a possibility for murine-cell infiltration and contamination while tumor grows in mice. It is recommended to begin with fresh PDX samples without necrotic regions and murine stromal cell infiltration.

2. Resuscitate the frozen lung PDX sample by incubating it in a 37°C water bath for 1 min.
3. In a biological safety cabinet (BSC), place a 60 mm culture dish on ice. Bring the thawed lung PDX sample to the culture dish with 5 mL of complete culture media [RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution] to prevent the sample from drying.
4. Mince the PDX sample into 2–3 $mm^3$ pieces with a sterile surgical scalpel and keep it aside on ice.
5. Prepare anesthesia cocktail solution by mixing ketamine/xylazine/PBS.

*Note:* The usage doses of the anesthesia cocktail are ketamine 200 mg/kg and xylazine 10 mg/kg.

6. Anesthetize 6–8-week-old female NOD/SCID mouse by injecting the anesthesia cocktail 100 μL per 10 g of body weight.

*Note:* The anesthesia onset takes 3–5 min with 30–40 min duration.

7. Remove the hair of the right flank of NOD/SCID mouse with clippers.
8. Make a 10–15 mm skin incision on the shaved region with surgical scissors.
9. Secure enough space under the skin by pushing a trocar needle.
10. Load 3–5 pieces of PDX samples on the tip of stainless-steel trocar needle. Add 20 μL of Matrigel over the loaded tissues and inject subcutaneously into the NOD/SCID mice.

*Note:* Matrigel helps to improve the frequency of tumor formation.

11. Close the surgical region with a sterile suture.
12. Return the mouse to the cage and monitor the mouse until fully recovered.

*Note:* Maintain the PDX-implanted mouse in a specific pathogen-free (SPF) room at a stable temperature (22 ± 2°C) with ad libitum availability of standard chow and water and 12 h of light/dark cycle.

13. Remove the suture by 10–14 days after surgical intervention.
14. Observe the PDX tumor growth and wait until it reaches the appropriate size (<500 $mm^3$).

*Note:* The time to reach the endpoint can vary depending on the condition of the PDX sample, the initial amount of tissue implanted, and/or the condition of the mouse.

### Collection of lung PDX tumor from immunodeficient mouse and establishment of a primary cell line

Timing: 2 h

In this section, we describe the processes of harvest and dissociation of PDX tumor for obtaining a primary cell line.

15. Anesthetize the PDX-bearing mouse by injecting an anesthesia cocktail of 100 μL per 10 g of body weight.
16. Shave around the PDX tumor with clippers and disinfect the surgery area with a 70% ethanol cotton swipe.
17. Dissect the flank skin and detach the PDX tumor.

⚠ CRITICAL: It is strongly recommended to perform this procedure in a BSC with sterile surgical tools and materials to prevent possible contamination.

⚠ CRITICAL: Remove the mouse dermis and necrotic parts completely from the tumor. It is important to secure viable tumor cells and to increase the yield of PDX primary cells.

*Note:* Surgical tools can be sterilized by autoclave sealed with an instant sealing sterilization pouch.

18. Place the collected PDX tumor in5 mL of complete culture media in a 15 mL conical tube for temporary storage or transport. Keep the sample in ice (4°C).

*Note:* Cutting into smaller pieces in this step may help to keep the sample submerged in the culture media preventing it from drying out.

19. In BSC, place the PDX tumor in a 60 mm culture dish on ice with 5 mL of complete culture media. Mince the PDX tumor into several small pieces of ~2–4 mm with a sterile scalpel.

*Note:* Chopping the PDX tumor into smaller pieces increases the efficacy of tumor dissociation enzymes and the yield of single cells.

20. Prepare the human Tumor Dissociation Kit (Miltenyi Biotec) component enzymes (Enzymes H, R, and A) by adding culture media (RPMI 1640) according to the manufacturer's instructions (Table 1). For storage, prepare aliquots of the enzyme solutions with proper volume to avoid repeated freeze-thaw cycles.

*Note:* Please refer to the manufacturer's protocol.

https://www.miltenyibiotec.com/upload/assets/IM0002061.PDF.

*Note:* 60 μL of Collagenase D (100 mg/mL) and 75 μL of DNase I (1% solution) in 3 mL of PBS can be used as alternative enzymes for tumor dissociation.

[troubleshooting 1].

**Table 1. Enzyme reagent preparation**

| Reagent | RPMI 1640 media amount | Aliquot amount |
|---|---|---|
| Enzyme H | 3 mL | 100 μL |
| Enzyme R | 2.7 mL | 50 μL |
| Enzyme A | 1 mL | 12.5 μL |

*Prepare the enzymes in BSC with sterile culture media. Aliquot the enzyme with an appropriate amount and store it at −20°C. These enzyme solutions are stable for up to 6 months after reconstitution.

21. Transfer the chopped PDX tumor into a gentleMACS™ C tube and add 2.2 mL of RPMI 1640 media, 100 μL of Enzyme H, 50 μL of Enzyme R, and 12.5 μL of Enzyme A.
22. Attach the C tube to the gentleMACS™ dissociator and run program h_tumor_01.
23. Detach the C tube and incubate in a 37°C $CO_2$ incubator for 30 min while inverting C tube every 5 min.
24. Attach the C tube to the gentleMACS™ dissociator and run program h_tumor_02 ($1^{st}$ round) (Tables 2 and 3).

*Note:* Depending on the PDX tumor type, select the appropriate program. The classification is based on the histological composition of the tissue.

25. Detach the C tube and Incubate in a 37°C $CO_2$ incubator for 30 min while inverting C tube every 5 min.
26. Attach the C tube to the gentleMACS™ dissociator and run program h_tumor_02 ($2^{nd}$ round).

*Note:* Run the appropriate gentleMACS program according to the Table 4.

27. Detach the C tube and centrifuge at 300 × *g* for 1 min at room temperate (RT, 22 ± 2°C) to collect the dissociated cells.
28. Resuspend the dissociated cell pellet with 10 mL of culture media (complete RPMI 1640) and transfer the cell suspension into a cell strainer (70 μm) placed on a 50 mL conical tube. Add 10 mL of culture media (complete RPMI 1640) to wash cells through the strainer.
29. Centrifuge the filtered cell suspension at 300 × *g* for 7 min. Remove the supernatant completely and resuspend the cell pellet in 5 mL of cell culture media (complete RPMI 1640).

*Note:* (optional) If the cell suspension is viscous, add DNase at 200 U/mL and incubate for 5 min at RT.

30. Add 100 μL of 0.4% trypan blue solution to 100 μL cell suspension.
31. Load 10 μL of cell suspension on a hemocytometer and count a viable (unstained) cell number.
32. Seed 0.5–1 × $10^7$ viable cells per a 90 mm culture dish with 10 mL complete culture media. Incubate cells at 37°C in a 5% $CO_2$ incubator for ~12–24 h.

*Note:* If you want to establish a primary PDX cell line, maintain these dissociated cells with subculture every 4–5 days.

[troubleshooting 2].

**Table 2. Classification of tumor types according to the histological tissue composition**

| Tumor type | Example |
|---|---|
| Soft | Melanoma, ovarian, colon, hypopharyngeal, or renal tumors |
| Medium | Lung and prostate tumors |
| Tough | Breast, pancreatic, hepatocellular, or head and neck squamous cell (HNSCC) tumors |

**Table 3. 1st round of gentleMACS programs for each tumor type**

| Tumor type | gentleMACS program |
|---|---|
| Soft | h_tumor_02 |
| Medium | h_tumor_02 |
| Tough | h_tumor_01 |

## CFSE staining

Timing: 60 min

In this section, we describe the process of CFSE staining for distinguishing SCCs.

33. Remove unattached dead cells and floating murine-immune cells with 5 mL of PBS (for a 90 mm culture dish) twice.
34. Add 1 mL of 2× TE solution to detach the viable cells from the culture dish. Incubate the cells in 37°C $CO_2$ incubator for 5 min.

*Note:* Sometimes the primary culture cells are more firmly attached to the bottom of the cell plate. As such, increase the incubation time with TE buffer up to 10 min.

35. Add complete culture media of two times the volume of 2× TE solution. Transfer the detached cells to a 15 mL conical tube and centrifuge at 300 × *g* for 7 min at RT.
36. Remove the supernatant and resuspend and wash the cell pellet with 5 mL PBS. Centrifuge at 300 × *g* for 7 min at RT.
37. Remove the supernatant and resuspend the cells at 2–20 × $10^7$ cells/mL in sterile PBS.

*Note:* It is recommended for a researcher to determine how many cells will be stained depending on the purpose of downstream analyses.

*Note:* Take 1 × $10^5$ cells and seed them in a 90 mm culture dish for CFSE unstained control cells.

38. Prepare 5 mM CFSE stock solution by adding 36 μL of anhydrous DMSO to 100 μg of lyophilized CFSE.

*Note:* Please refer to the manufacturer's protocol.

CFSE Cell Division Tracker Kit (d1spbj2x7qk4bg.cloudfront.net).

39. Prepare 2× concentration of CFSE working solution (10–200 μM) by diluting the stock solution in the same volume of PBS for cell resuspension.
40. Mix well the cell suspension with 2× CFSE working solution to stain the cells.
41. Incubate the cells at 37°C, 5% $CO_2$ incubator for 20 min. Tap the staining tube every 5 min for mixing (Figure 1).

*Note:* Keep the cells protected from light.

**Table 4. 2nd round of gentleMACS programs for each tumor type**

| Tumor type | gentleMACS program |
|---|---|
| Soft | h_tumor_03 |
| Medium | h_tumor_02 |
| Tough | h_tumor_01 |

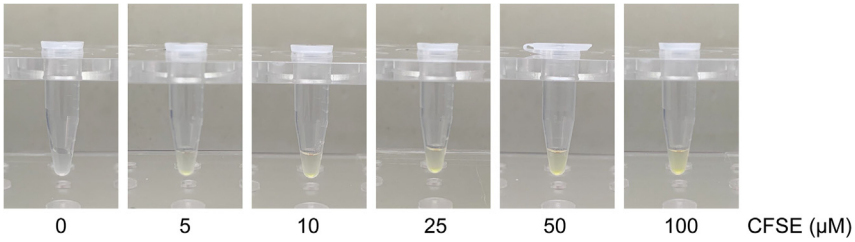


**Figure 1. CFSE staining with increasing concentration**
Photograph of the cells with 0, 5, 10, 25, 50, and 100 μM CFSE staining at 37°C for 20 min.

⚠ CRITICAL: High concentration of CFSE is toxic for cells. Determine the ideal CFSE staining concentration based on the cell number and growth rate compared to the unstained control.

42. Quench the CFSE staining by adding 10% FBS-containing complete culture media holding at least 5 times the staining volume.
43. Centrifuge at 300 × *g* for 7 min. Remove the supernatant completely and resuspend the cells with fresh complete media.
44. Seed 1–5 × $10^5$ cells per a 90 mm culture dish for up to 5 days (Figure 2).

*Note:* Optimal incubation period can differ depending on the proliferation rate of the stained cells. It should be determined by the researcher. Determine the incubation end point before ~50%–60% of cells lose their fluorescence during proliferation. Observation under the fluorescent microscope helps deciding the incubation endpoint.

[troubleshooting 3].

[troubleshooting 4].

## Cell sorting

Timing: 2 h

In this section, we describe the process of flow cytometry cell sorting to obtain human cancer cells and the isolation of cancer cell subpopulations depending on their proliferation rates.

45. Detach the stained cells with 1 mL of 2× TE buffer. Incubate the cells in 37°C $CO_2$ incubator for 5 min. After adding 2 mL of complete culture media collect the detached cells in a 15 mL conical tube.
46. Centrifuge at 300 × *g* for 7 min.
47. Remove the supernatant and resuspend the cell pellet with 5 mL PBS for washing.
48. Centrifuge at 300 × *g* for 7 min.
49. Remove the supernatant and resuspend the pellet with 3 mL PBS.
50. Load 10 μL of cell suspension on hemocytometer for cell counting.
51. Take a desirable number of CFSE-stained cells and transfer them to a new sterile 1.5 mL microcentrifuge tube.

*Note:* Decide the cell number for following cell sorting based on how many cells the researcher requires for downstream experiments.

*Note:* In this protocol, the final $CFSE^{high}$ or $CFSE^{low}$ populations would be about 5% of total cell number.

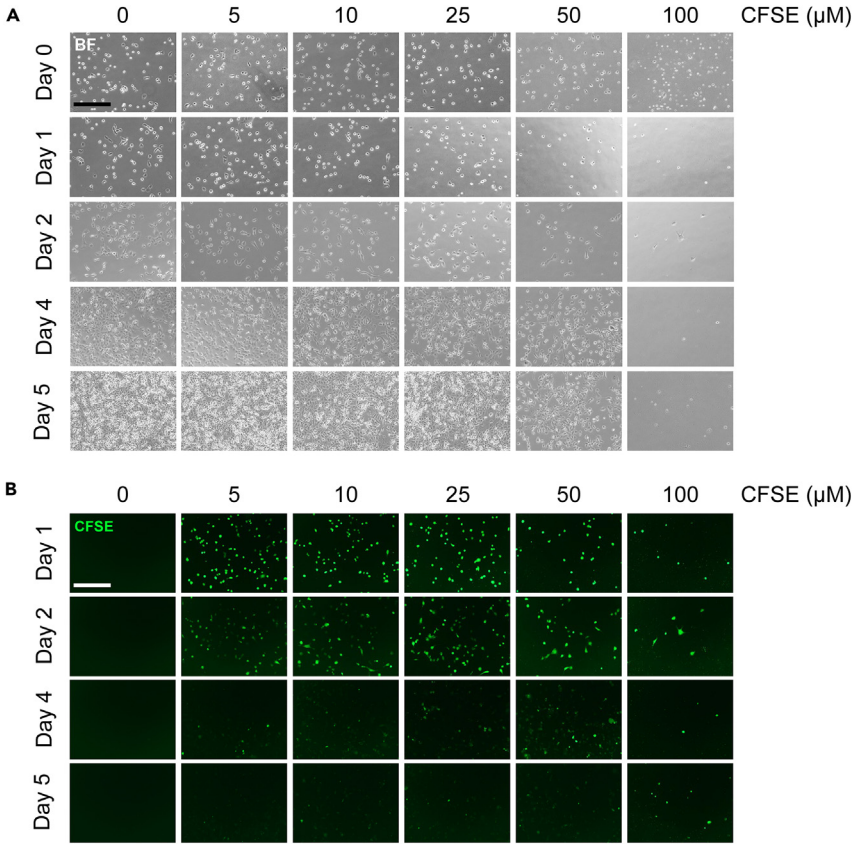


**Figure 2. Photograph of brightfield and CFSE fluorescence with different CFSE staining concentrations until Day 5**

(A and B) Photograph of brightfield (A) and CFSE fluorescence (B) with different CFSE staining concentrations until Days 5. CFSE-stained primary culture cells in Figure 1 were seeded in 6-well plates ($10^5$ cells/well) and incubated until Day 5. After day 0, floating dead cells were removed by media change prior to photography. Representative brightfield and CFSE fluorescent photographs from each condition were shown. Scale bar: 400 μm. BF: bright field

*Note:* Transfer 1–5 × $10^5$ cells into a new 1.5 mL microcentrifuge tube a APC FMO control (CFSE-stained only).

52. Centrifuge at 300 × *g* for 7 min.
53. Remove the supernatant.
54. Resuspend the cell pellet with sterile flow cytometry buffer at $10^6$ cells/100 μL.

*Note:* Flow cytometry buffer (0.5% BSA, 2 mM EDTA in PBS) should be sterilized by filtration (0.22 μm).

*Note:* For the unstained control cells, resuspend the cells with the same volume of staining tube.

55. Stain the cells with murine CD45-APC, CD31-APC, and PDGFRα-APC antibodies (1:200, v/v) at 4°C for 30 min.

*Note:* Keep the cells protected from light.

56. Add 1 mL flow cytometry buffer and centrifuge at 300 × *g* for 7 min at 4°C.
57. Remove the supernatant and wash the cell pellet with 1 mL flow cytometry buffer.

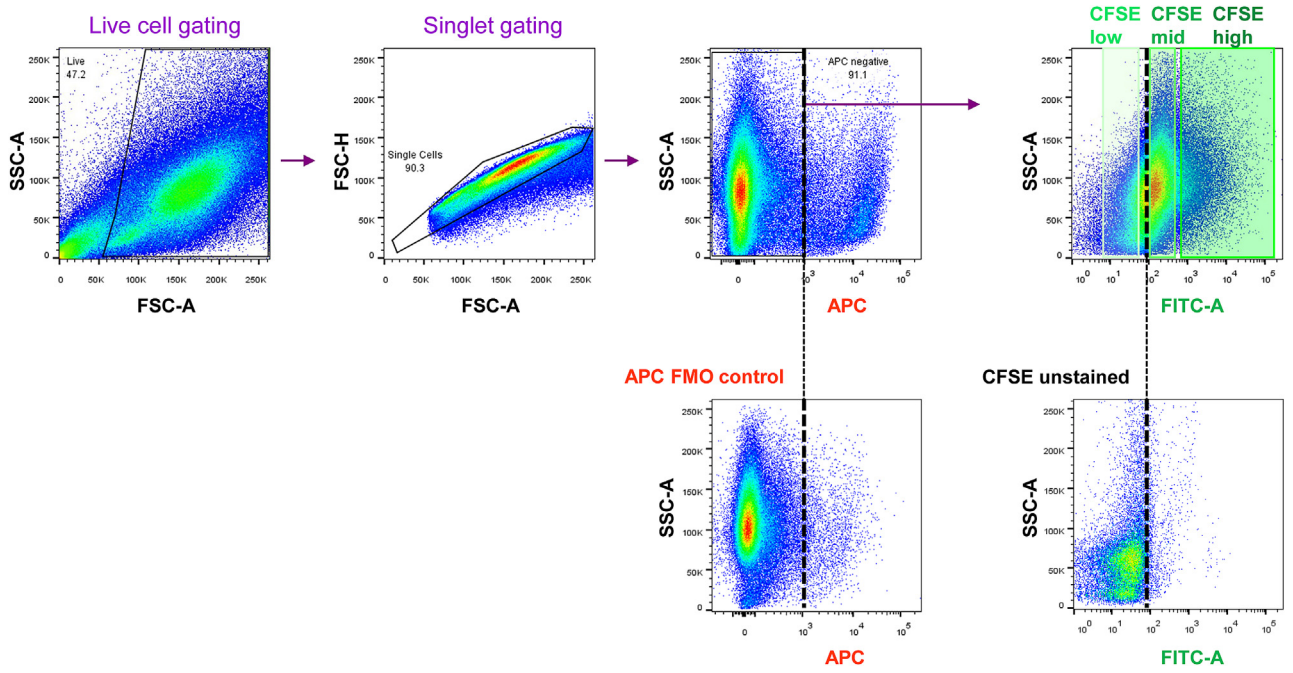


**Figure 3. Gating strategy for sorting of CFSE$^{low}$, CFSE$^{mid}$, and CFSE$^{high}$ populations from primary culture cells.**

58. Centrifuge at 300 × *g* for 7 min.
59. Remove the supernatant and wash the cell pellet with 1 mL flow cytometry buffer one more time.
60. Centrifuge at 300 × *g* for 7 min.
61. Remove the supernatant completely. Resuspend the cell pellet with flow cytometry buffer at 1–4 × $10^6$ cells/mL concentration.

⚠ CRITICAL: Cell concentration is important to achieve efficient cell separation. The recommended cell concentration is 0.5–1 × $10^6$ cells in 250–1,000 μL flow cytometry buffer.

62. Transfer the resuspended cells into the FACS tube strainer cap.

*Note:* FACS tubes and their strainer caps should be sterile to avoid contamination. Always perform this procedure on a BSC.

63. Sort the cells with the FACS Aria cell sorter III (Figure 3).
    a. Draw a gate for live cell populations (y-axis: SSC-A, x-axis: FSC-A).
    b. Draw a linear gate for a single cell population (y-axis: FSC-H, x-axis: FSC-A).
    c. Select APC-negative cell populations, which represent human tumor cells (APC-positive cells represent murine immune cells, endothelial cells, and stromal cells; y-axis: SSC-A, x-axis: APC-A).

*Note:* Draw an APC-negative gate based on APC FMO control (CFSE-stained only).

[troubleshooting 5].

    d. Divide the cell populations into three regions: FITC high, FITC middle, and FITC low. Each population represents cancer cells with low, middle, and high proliferation rates (y-axis: SSC-A, x-axis: FITC-A).

*Note:* Draw a bottom line of CFSE$^{mid}$ upper than CFSE unstained.

*Note:* We expect to obtain the sorted populations up to 50% of viability.

*Note:* The recommended threshold for determining CFSE$^{low,}$ CFSE$^{mid}$, and CFSE$^{high}$ are bottom 10%, middle 30%–40%, and upper 10% of APC-negative population.

[troubleshooting 6].

64. Prepare sterile 15 mL conical tubes as collection reservoirs with 6 mL complete cell culture media.

*Note:* To increase the yield of sorted cells, wet the walls of the collection tube with cell culture media before sorting.

65. Collect the sorted cells in sterile collection tubes.
66. Centrifuge the sorted cells at 300 × *g* for 7 min.
67. Remove the supernatant.
68. Resuspend the sorted cells with 5 mL of fresh culture media.
69. Centrifuge at 300 × *g* for 7 min.
70. Resuspend the cells with 3–5 mL of culture media depending on the cell pellet size.

### Cell proliferation assay

Timing: 4–5 days

In this section, we describe an assay to evaluate the proliferation rates of CFSE-sorted cells.

71. Seed 200 cells of CFSE$^{high}$, CFSE$^{mid}$, and CFSE$^{low}$ in 12 well plates (200 cell/1 mL culture media).

[troubleshooting 7].

72. Incubate 12 well plate in a 37°C 5% $CO_2$ incubator for 4 days.
73. Aspirate cell culture media and wash cells with 1 mL PBS.
74. Remove PBS and wash cells with 1 mL PBS one more time.
75. Remove PBS completely.
76. Fix the cells with 100% methanol for 10 min at RT.
77. Aspirate the methanol from the well.
78. Stain cells with 0.5% crystal violet solution for 1 h at RT.
79. Remove the staining solution.
80. Wash remained staining solution with 3 mL of distilled water three times.
81. Analyze stained CFSE$^{low}$, CFSE$^{mid}$, and CFSE$^{high}$ cells.

*Note:* Refer to the Cho et al., 2021 (Ref. #[1]) publication for more *in vitro* and *in vivo* experiments comparing cellular properties among CFSE$^{low}$, CFSE$^{mid}$, and CFSE$^{high}$.

## EXPECTED OUTCOMES

To improve the therapeutic efficacy and the survival rate of cancer patients, it is crucial for investigating the mechanism of anticancer drug resistance and tumor recurrence. To this end, it is required to obtain slow-cycling, dormant cancer cells that play an important role in anticancer drug resistance and tumor recurrence by separating tumor cells depending on their proliferation rates. This protocol describes the procedures for establishing a primary cell line of a lung PDX tumor from an immunodeficient mouse and sorting their ACC and SCC populations using CFSE fluorescence retention that

decreases with cellular division. The sorted subpopulations of slow, middle, and fast-cycling cancer cells can be used for further analyses to verify their different proliferation abilities, explore the changed mRNA expression among these cells, and test their capacity for tumor formation in *in vivo* experiments. The separated cells can also be used for gene expression profiling analysis with next-generation sequencing technologies.[1]

Regarding the establishment of lung PDX primary cells, we used a human Tumor Dissociation kit (Miltenyi Biotec) to isolate primary tumor cells from the lung PDX tumor of low passage number (less than 5). The isolated primary cells include viable PDX tumor cells, a little portion of infiltrated murine immune cells and stromal cells. With this protocol, we expect to obtain 1–5 × $10^7$ total cells per tumor gram on average with ~40% of viability. The viable PDX tumor cells can be isolated by 1) continuous subcultures for a long period or 2) applying murine cell components specific antibodies and sorting out the antibody's negative population.

CFSE is a green fluorescent cell staining dye that incorporates cellular lysine residues and amines. The retention of this dye inside the cell can be used as an indicator of cellular proliferation. By adopting this trait we tried to segment the PDX primary cells into $CFSE^{low}$ (ACCs), $CFSE^{mid}$, and $CFSE^{high}$ (SCCs) populations. Instead of CFSE, red staining dye PKH26, which has a similar principle of dilution, was able to be used with this protocol and resulted in similar outcomes.[1,4]

Regarding the assessment of properties of the SCCs and ACCs populations, we adopted various assays including comparing cellular proliferation, Ki67 positivity, and mitotic index, and also investigated time-dependent *in vivo* tumor incidence (Figure 4).[1] These approaches verified that the population of low dye retention ($CFSE^{low}$, ACCs) showed the properties of fast-growing cells that were more sensitive to chemotherapy and that the population of high dye retention ($CFSE^{high}$, SCCs) recapitulates a chemotherapy-resistant, dormant-like phenotype.[1]

## LIMITATIONS

In this protocol, we suggested an efficient method to isolate slow-cycling cancer cells (SCCs) of lung PDX from an immunocompromised mouse. A tumor is a sum of diverse heterogenous populations, which includes populations with different cellular properties. SCCs contribute to tumor metastasis, recurrence, and the development of drug resistance.[5] It has been hard to obtain pure SCCs from the bulk of tumor cells, which has been a major obstacle in studying the biology of SCCs. In addition, despite the crucial role of dormant SCCs in metastasis, the metastatic ability of SCCs could have not been appropriately evaluated due to the absence of proper experimental models.

Patient-derived xenograft (PDX) is a promising specimen to investigate human tumors in that maintain intra-tumor heterogeneity. However, because of the nature of fragment passaging, studying SCCs biology with PDX is exposed to a risk that some inherent SCCs populations may not be studied in the fraction under investigation. In addition, as the passage number grows, the composition of the tumor cell population in PDX can be altered with subpopulations selected from growth periods. To reduce this risk, it is recommended to use an early passage of PDX tissue as much as possible and prepare the passaging sample with a mixture of several small PDX fragments to maintain the heterogeneity. Accumulating evidence suggests that tumor cells have plasticity,[6] and this makes it hard to establish a reliable *in vitro* model for studying SCCs. This limitation still exists in our protocol. Maintaining the dissociated PDX primary cells before and after CFSE staining in the 2D culture system would instigate the changes in cell cycle properties. Indeed, while keeping the sorted ACCs and SCCs on the culture dishes, it was observed that the differences between the two groups, such as proliferation rate and drug resistance, gradually decreased. This phenomenon may be attributed to the imperfection of cell sorting mediated by CFSE staining, the plasticity of tumor cells during the cell culture or an artificial culture conditions such as plat bottom plate, abundant nutrients, and ample amounts of ligands for cell proliferation. However, compared to other experimental

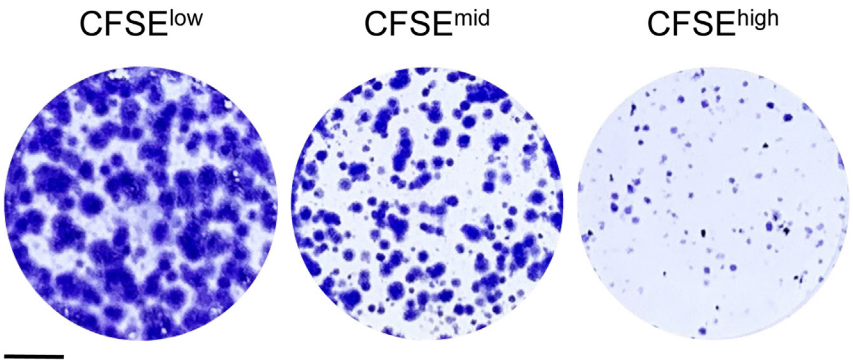


**Figure 4. Different proliferation rates of CFSE$^{low}$, CFSE$^{mid}$, and CFSE$^{high}$ lung PDX primary cells**
200 cells were seeded in 12-well plates, and proliferated cells were fixed and stained with crystal violet on Day 4. Scale bar: 5 mm.

options for example organoid culture or reinjection into an immunocompromised mouse for xenograft after CFSE staining, our protocol allows investigating the intrinsic cell properties of SCCs with simple procedures and abundant amounts for downstream analyses. In addition, the CFSE-mediated sorted cells from our protocol showed consistent and reliable differences among the groups within early passage numbers.

## TROUBLESHOOTING

### Problem 1

Tumor dissociation is insufficient (step 20).

### Potential solution

For the dissociation of the tumor larger than 0.2g increase the volume of media and enzymes two times (4.7 mL culture media, 200 μL Enzyme H, 100 μL Enzyme R, 25 μL Enzyme A). Increase the incubation time in steps 23 and 25.

### Problem 2

After the isolation of PDX primary cells, the number of viable cells is too low (step 32).

### Potential solution

When preparing the lung PDX tumor sample, completely remove the mouse dermis on the tumor's outer surface and necrotic parts inside of the tumor mass as much as possible. Starting the dissociation step with the tumor less than 500 $mm^3$ (without necrotic regions) is beneficial for increasing the yield of viable primary cells.

### Problem 3

CFSE staining is too weak to distinguish ACCs and SCCs after the incubation period (step 44).

### Potential solution

Perform a pilot study with various staining concentrations and incubation times with a small number of cells. Increase the CFSE staining concentration up to a value below cell toxicity or shorten the incubation time to 2–3 days.

### Problem 4

After CFSE staining the cell viability is too low (step 44).

### Potential solution

The high concentration of CFSE staining is toxic to the cell. Decrease the concentration of CFSE staining.

### Problem 5

After flow cytometry cell sorting, murine cell populations were not excluded completely (step 63).

### Potential solution

Make a sorting gate based on proper control cells (CFSE-stained only. Unstained with APC conjugated antibodies). Draw the sorting gate more rigorously. Addition of staining with antibodies targeting markers expressed exclusively on human tumor cells such as human EpCAM or human CD298 would increase the purity of human origin.

### Problem 6

The viability of $CFSE^{low}$ is too low (step 63).

### Potential solution

Do not include cells with too low values among the FITC positives. This population could contain dead cells or cell debris. Staining with a cell viability dye (e.g., 7-AAD, CyTRAK Orange, or zombie dye) can be used to exclude dead cells.

### Problem 7

The number of cells recovered after CFSE sorting is too low compared with the number FACS Aria showed (step 71).

### Potential solution

Transfer the sorted cell suspension to a container of smaller volume such as several 1.5 mL microcentrifuge tubes and centrifuge. It is easier to distinguish cell pellets.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ho-Young Lee (hylee135@snu.ac.kr).

### Materials availability

This study did not generate any unique reagents.

### Data and code availability

This study did not generate any datasets and code.

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## AUTHOR CONTRIBUTIONS

Conceptualization, H.Y.L.; methodology, J.C., H.Y.L.; formal analysis, J.C.; investigation, J.C.; writing – original draft, J.C.; writing – review & editing, J.C., H.Y.M., H.Y.L.; visualization, J.C.; supervision, H.Y.L.; funding acquisition, H.Y.L.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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