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Protocol for tumor dissociation and fluorescence-activated cell sorting of human head and neck cancers

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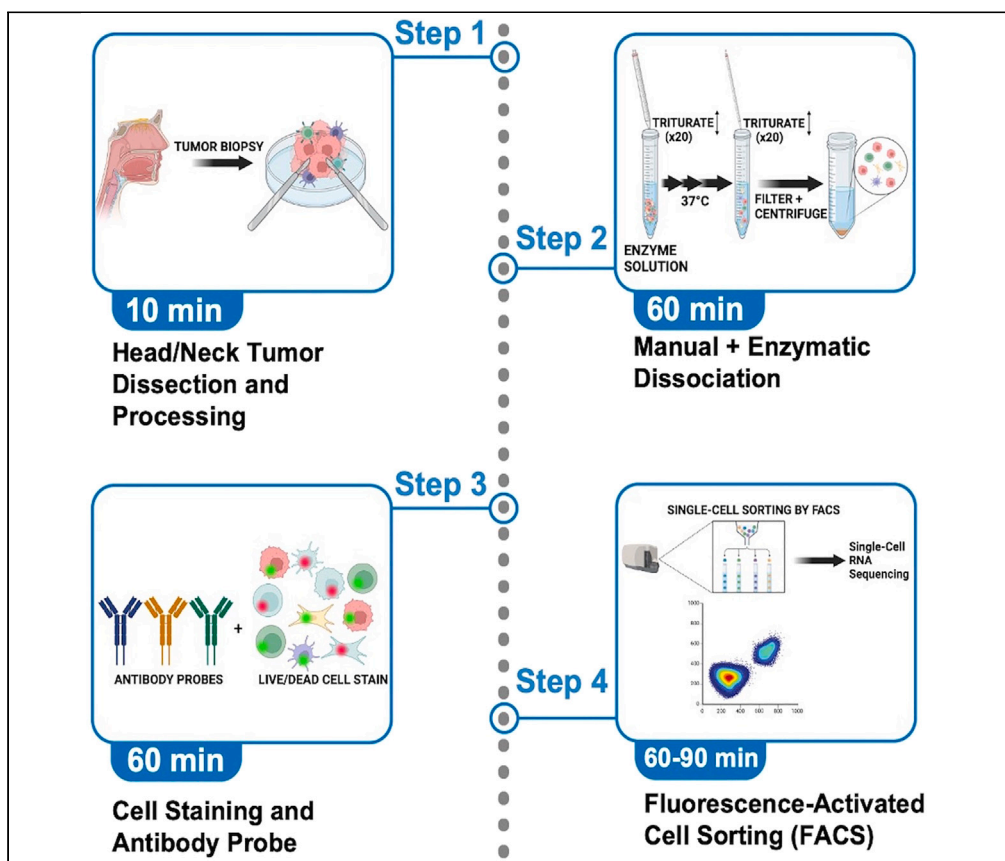
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Protocol

Protocol for tumor dissociation and fluorescence-activated cell sorting of human head and neck cancers



Tumors originating from the head and neck represent diverse histologies and are comprised of several cell types, including malignant cells, cancer-associated fibroblasts, endothelial cells, and immune cells. In this protocol, we describe a step-by-step approach for the dissociation of fresh human head and neck tumor specimens, followed by isolation of viable single cells using fluorescence-activated cell sorting. Our protocol facilitates the effective downstream use of techniques, including single-cell RNA sequencing and generation of three-dimensional patient-derived organoids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights
Mechanical and enzymatic dissociation of fresh human head and neck tumor biopsies

FACS to isolate viable single cells and enrich for malignant cells and T lymphocytes

Applicable for squamous cell carcinoma and adenoid cystic carcinoma tissue samples

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Protocol

Protocol for tumor dissociation and fluorescence-activated cell sorting of human head and neck cancers

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SUMMARY

Tumors originating from the head and neck represent diverse histologies and are comprised of several cell types, including malignant cells, cancer-associated fibroblasts, endothelial cells, and immune cells. In this protocol, we describe a step-by-step approach for the dissociation of fresh human head and neck tumor specimens, followed by isolation of viable single cells using fluorescence-activated cell sorting. Our protocol facilitates the effective downstream use of techniques, including single-cell RNA sequencing and generation of three-dimensional patient-derived organoids.

For complete details on the use and execution of this protocol, please refer to Puram et al. (2017)¹ and Parikh et al. (2022).²

BEFORE YOU BEGIN

This protocol describes in detail the necessary steps for the dissociation of head and neck tumor samples and fluorescence-activated cell sorting (FACS) to isolate singlet viable cells and enrich for malignant cells or T lymphocytes for single-cell transcriptomic analysis or generation of 3D patient-derived organoids (PDO). We have used this protocol to examine the expression heterogeneity landscape of head and neck squamous cell carcinoma (HNSCC) and head and neck adenoid cystic carcinoma (ACC).^{1,2}

Institutional permissions

Before the initiation of this protocol, human studies must be approved by an institutional review board (IRB). Researchers must collect patient samples in concordance with the IRB guidelines, including proper recruitment, informed consent, and minimal-risk procurement.



Preparation for tumor dissociation

⌚ Timing: 40 min

1. Prepare PBS, 1% BSA stock.
 - a. Add 0.2 g of BSA in 20 mL Dulbecco's Phosphate buffered saline (DPBS). Store at 2°C–8°C for up to 1 week.
2. Prepare RBC Lysis Solution (10%):
 - a. Add 1 mL RBC solution to 9 mL ddH₂O. Store protected from light at 2°C–8°C (do not freeze).
3. Prepare Enzyme H according to manufacturer specifications³:
 - a. Reconstitute lyophilized powder in 3 mL Roswell Park Memorial Institute (RPMI) 1640.
 - b. Prepare aliquots of 200 μL each in a 1.7 mL microcentrifuge tube to avoid freeze-thaw cycles. Store at –20°C.
4. Prepare Enzyme R according to manufacturer specifications³:
 - a. Reconstitute lyophilized powder in 2.7 mL RPMI 1640.
 - b. Prepare aliquots of 100 μL each in a 1.7 mL microcentrifuge tube to avoid freeze-thaw cycles. Store at –20°C.
5. Prepare Enzyme A according to manufacturer specifications³:
 - a. Reconstitute lyophilized powder in 1 mL Buffer A (supplied with kit).
 - b. Do not vortex. Prepare aliquots of 25 μL each in a 1.7 mL microcentrifuge tube to avoid freeze-thaw cycles. Store at –20°C.

Note: If this protocol is being performed for organoid or cell culture, all steps must be performed under sterile conditions in a tissue culture hood. Furthermore, 1% BSA stock and reconstituted enzymes H, R, and A must be sterile-filtered through a 0.22 μm membrane before use. If organoid or cell culture is not intended, all steps can be performed under RNase-free laboratory conditions.

Note: Mix suspension thoroughly to ensure that the powder is dissolved before aliquoting. Avoid vortexing.

Note: Per manufacturer specifications, enzyme solutions are stable for six months after reconstitution.³ However, in our experience, solutions stored at –20°C may be used for up to one year after reconstitution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal mouse CD45-ivoblu (clone:5B1) 1:10 dilution	Miltenyi Biotec	Cat#130-092-880
Monoclonal mouse CD90-PE (clone 5E10) 1:20 dilution	BD Biosciences	Cat#555596
Monoclonal mouse CD31-PE-cy7 (clone WM59) 1:20 dilution	BD Biosciences	Cat#563651
Monoclonal mouse CD3-PE-cy7 (clone UCHT1) 1:20 dilution	Thermo Fisher Scientific	Cat#25-0038-42
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate buffered saline (DPBS)	Thermo Fisher Scientific	Cat#14190250
Human tumor dissociation kit (Enzyme H, R, A)	Miltenyi Biotec	Cat#130-095-929
Bovine serum albumin (BSA)	Sigma-Aldrich	Cas#9048-46-8
Red blood cell (RBC) lysis solution (10×)	Miltenyi Biotec	Cat#130-094-183
Calcein, AM, cell-permeant dye 1:1000 dilution	Life Technologies	Cat#C3100MP

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TO-PRO-3 iodide 1:3000 dilution	Life Technologies	Cat#T3605
Roswell Park Memorial Institute (RPMI) 1640 Medium	Thermo Fisher Scientific	Cat#11-875-093
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	Cat#11885084
Buffer TCL	Qiagen	Cat#1031576
Beta-Mercaptoethanol	Sigma-Aldrich	Cat#M6250-100ML
Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific	Cat#14025092
Other		
15 mL/50 mL Conical centrifuge tubes	Corning Life Sciences DL, Fisher Scientific	Cat#339650 (15 mL) Cat#339650 (50 mL)
Fisherbrand Cell Strainers. Nylon mesh 100 μ m	Thermo Fisher Scientific	Cat#22-363-549
Petri dishes	Corning	Cat#353003
Sterile scalpels	Exel International	Cat#29550
5 mL, 10 mL, 25 mL serological pipettes	Thermo Fisher Scientific	Cat#170355, 170356, 170357
Eppendorf 1.7 mL tubes	DOT Scientific Inc	Cat#RN1700-GMT
LoBind Eppendorf Tubes	Eppendorf	Cat#022431021
Falcon 5 mL test tubes, with Cell 35 μ m strainer cap (FACS tubes)	Corning	Cat#352235

STEP-BY-STEP METHOD DETAILS

Preparation of enzyme mix

⌚ **Timing:** 20 min before initiating protocol

1. Pre-warm a 4.7 mL aliquot of RPMI or Dulbecco's Modified Eagle Medium (DMEM) in a 15 mL conical tube using a 37°C incubator.
2. Thaw a frozen aliquot of enzyme H, R, and A on room temperature or on ice.
3. Prepare enzyme mix by adding 200 μ L Enzyme H, 100 μ L Enzyme R, and 25 μ L enzyme A to 4.7 mL pre-warmed RPMI 1640 or DMEM in a 15 mL conical tube.

Note: This volume of enzyme mix is optimized for tumor samples that weigh up to 1 g of tissue or are $\sim 0.5 \times 0.5$ cm in area. For larger samples, double the volume of enzyme mix.

Mincing and washing of head and neck tumor specimen

⌚ **Timing:** 10 min

4. On a dry petri dish at room temperature, use two sterile scalpels to remove and discard fat, fibrous tissue, clots, and necrotic areas from the tumor sample.

Note: Tumor tissue is firm and white or pink in appearance; adipose tissue is soft and yellow in appearance; fibrous tissue appears white with a rubbery texture; clots are soft and appear purple or black; necrotic tissue is soft and are typically brown or black in appearance.

5. Thoroughly mince the tumor tissue sample into approximately 1–2 mm pieces (Figure 1).

⚠ CRITICAL: Ensure that the tumor sample is thoroughly minced, as incomplete mincing may lead to diminished cell yield.

6. Transfer as much of the minced sample to a 15 mL conical tube as possible using a scalpel.
7. Add 10 mL PBS to the petri dish and pipette it over the surface of the petri dish multiple times to collect any residual tissue fragments. Transfer the PBS solution containing residual tissue fragments to the 15 mL conical tube.

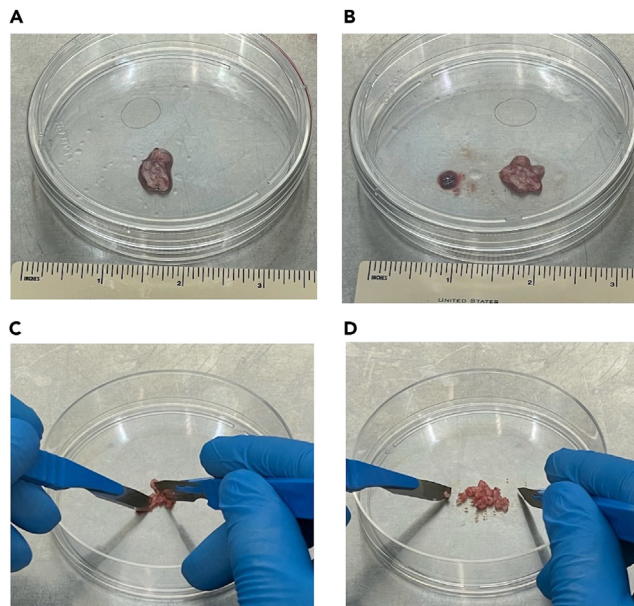


Figure 1. Mincing of human head and neck tumor tissue

(A) Initial tumor sample.
(B) Tumor sample after the removal of fatty, fibrous, hemorrhagic, and necrotic areas.
(C and D) Mincing of tumor tissue into 1–2 mm pieces using two sterile scalpels.

8. Centrifuge the 15 mL conical tube containing the sample for 5 min at 300 g at 4°C.

Mechanical and enzymatic dissociation of head and neck tumor specimen

⌚ Timing: 60 min

9. After centrifuging, aspirate and discard supernatant and add pre-warmed enzyme mix.
10. Triturate (pipette up and down approximately 20 times) with a 10 mL serological pipette (Figure 2A).

Note: If the specimen is larger (i.e., greater than 2 g or 1 cm³), consider first triturating the sample using a 25 mL pipette in a 50 mL conical tube.

△ CRITICAL: When triturating, titrate technique to the ease with which the sample is aspirated into the pipette. In the early stages of dissociation, when tissue fragments are larger, there may be difficulty with pipetting fragments. If this is encountered, stop triturating and proceed to the incubation step. Forcing the sample into the pipette when it is too large may result in loss of sample into the pipette filter due to buildup of negative pressure or difficulty with expelling it out of the pipette, causing buildup of positive pressure and loss of sample out of the top of the conical tube.

11. Incubate the sample in a water or bead bath at 37°C for 10 min and invert the tube every few minutes to maximize surface area of sample exposed to the enzyme mix.
12. Repeat steps 10–11 to effectively dissociate the sample. When possible, switch to triturating with a 5 mL serological pipette.

Note: As the sample begins to dissociate, it will be possible to pipette up and down with the pipette tip close to or in contact with the bottom of the conical tube (Figure 2B). This

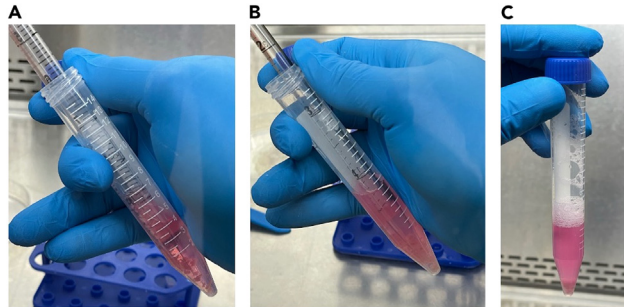


Figure 2. Trituration of head and neck tumor sample with serological pipette

- (A) Tumor sample prior to trituration.
 (B) Placement of serological pipette in contact with the bottom of the 15 mL conical tube to facilitate additional mechanical dissociation.
 (C) Sample after multiple rounds of trituration.

technique narrows the space through which the cell solution passes, thus creating additional mechanical shearing forces for dissociation of cell clusters.

△ CRITICAL: When tritulating with the pipette in contact with the bottom of the conical tube, it is critical to avoid buildup of negative or positive pressure in the pipette, which may result in loss of sample as described above.

- As the sample dissociates, the media should start to appear cloudy. To assess the adequacy of dissociation, remove 90 μ L sample into a 1.7 mL microcentrifuge tube and add 10 μ L trypan blue. Under the microscope, the sample should contain mostly individual cells; if the majority of the cells are clustered together, then the sample may benefit from additional trituration. Steps 10–11 should be repeated for no more than 1 h, as extended incubation in the enzyme mix may cause excessive cell death.

Note: For treatment-naïve head and neck squamous cell carcinoma (HNSCC) primary tumor samples, we find that approximately an hour of dissociation time is sufficient. For adenoid cystic carcinoma (ACC) samples, dissociation typically takes 30–45 min, as samples are less fibrous and more highly cellular. For lymph node samples, regardless of cancer type, dissociation also typically takes 30–45 min. For tumors samples that are large (greater than 1 cm³) and difficult to dissociate, consider dividing the original sample in two and performing two dissociations in parallel.

- After adequate dissociation, pipette the single cell suspension through a 100 μ m (yellow) cell strainer into a 50 mL conical tube. (Figure 3).

△ CRITICAL: Pipette the sample onto the bottom of the strainer, rather than onto the sides, as straining through the sides may result in loss of sample when removing the strainer.

- Wash the strainer with 10 mL media (RPMI 1640 or DMEM) and then wash the cells by pipetting up and down.

Note: Store the cell strainer containing residual tissue on ice or at room temperature. Residual tissue can be reprocessed with fresh enzyme solution using the same protocol if the cell recovery is low. The cell strainer can be discarded at the end of the dissociation once an adequate cell count is confirmed.

- Centrifuge the sample for 5 min at 300 g at 4°C.



Figure 3. Filtration of single cell suspension

A 100 µm strainer is used to filter gross debris and undissociated tissue.

Preparation and staining of single-cell suspension

⌚ Timing: 30–40 min

17. Aspirate and discard the supernatant. To avoid disrupting the pellet, the last 1 mL of supernatant may be removed carefully with a P1000 pipette.
 - a. At this point, if a tumor specimen is being processed for cell or organoid culture applications it may not be necessary to perform cell sorting prior to culture and the cell suspension can simply be resuspended in the appropriate culture media.
18. If the pellet appears red, it may contain red blood cells (RBCs). Remove these cells using RBC lysis solution. If the pellet appears white, proceed to step 19.
 - a. Resuspend the pellet in 1 mL Hanks' Balanced Salt Solution (HBSS).
 - b. Prepare 1× RBC lysis solution (1 mL RBC solution in 9 mL ddH₂O).
 - c. Add 9 mL 1× RBC lysis solution to 1 mL cell suspension in HBSS.
 - d. Vortex for 5 s.
 - e. Incubate for 5 min at room temperature.
 - f. Centrifuge for 5 min at 300 g at 4°C.
 - g. Aspirate and discard the supernatant.

Note: In our experience, careful separation of clots and bloody tissue in step 1 typically prevents RBC contamination.

19. Resuspend cells in PBS + 1% BSA such that the final volume is 100 µL after adding antibodies (step 20).
 - a. If multiple different cell populations are to be isolated by FACS, the dissociated specimen can be divided into separate 100-µL samples at this point.
20. Add 10 µL CD45-VioBlue, 5 µL CD90-PE, and 5 µL CD31-PE-Cy7 antibodies to the cell suspension.

Note: This combination of antibodies is most useful for enriching for malignant cells from HNSCC tumors by depleting for immune cells (CD45), fibroblasts (CD90), and endothelial

cells (CD31). Due to the higher fraction of malignant cells in ACC tumors, enriching CD90 negative and CD31 negative cells is typically not necessary.

Note: To enrich for T cells, stain with the combination of 10 μL CD45-VioBlue and 5 μL CD3-PE-cy7.

Note: Antibody-fluorophore complexes are sensitive to photo-oxidation. Protect these stocks from light by turning off the lights in the tissue culture hood while staining.

21. Incubate sample on ice covered with foil for 20 min.
22. Centrifuge the sample for 5 min at 300 g at 4°C. Remove the supernatant.
23. Wash cells with 1 mL PBS, 1% BSA.
24. Centrifuge the sample for 5 min at 300 g at 4°C. Remove the supernatant.
25. Re-suspend the cells in 0.5–1.5 mL PBS, 1% BSA (volume correlating with pellet size) and transfer the sample to a FACS tube with a 35 μm filter top. Pipette carefully through the filter. Change the top to non-filtered top. Keep the samples on ice protected from light by covering with foil to prevent photo-oxidation.

Fluorescence-activated cell sorting

⌚ Timing: 60–90 min

26. Prior to sorting, add calcein AM (1:1000) to stain live cells and To-Pro-3 iodide (1:3000) to stain dead cells.
 - a. For example, for a total volume of 150 μL , add 1.5 μL calcein and 0.5 μL To-Pro-3 iodide.
27. Gently vortex and allow 1 min for the stain to work.

Note: Add calcein and To-Pro-3 Iodide immediately prior to sorting. If multiple samples are being sorted, add compounds individually.

Note: Alternative live/dead cell stains can be used as long their spectra do not significantly overlap with the other antibodies used for sorting.

28. Sort as appropriate for subsequent applications.
 - a. For plate-based single-cell RNA-sequencing protocols, sort 1 cell per well of a 96-well plate prepared with 10 μL lysis buffer (buffer TCL + 1% beta-mercaptoethanol).
 - b. For droplet-based single-cell RNA-sequencing protocols, sort cells into a 1.7 mL Lobind Eppendorf microcentrifuge tube containing PBS with 1% BSA.
 - c. For cell and 3D organoid culture applications, cells may be sorted directly into serum-containing media in a 1.7 mL microcentrifuge tube.

Note: FACS must be performed on a FACSria Fusion Special Order System (BD Biosciences) or equivalent, using 488 nm (calcein AM, 530/30 filter), 640 nm (To-Pro-3, 670/14 filter), 405 nm (Vioblue, 450/50 filter), 561 nm (PE, 586/15 filter; PE-Cy7, 780/60 filter) lasers.

⚠ CRITICAL: A 100 μm nozzle must be used for cell sorting, as the 70 μm nozzle with higher sorting pressures results in frequent lysis of cells and decreased viability of sorted cells.

EXPECTED OUTCOMES

This protocol permits the successful isolation of malignant cells from HNSCC primary tumors (with sample FACS plots in [Figure 4](#)), metastatic HNSCC lymph nodes ([Figure 5](#)), and ACC primary tumors ([Figure 6](#)). Singlet viable cells can be captured using forward scatter height versus area criteria to

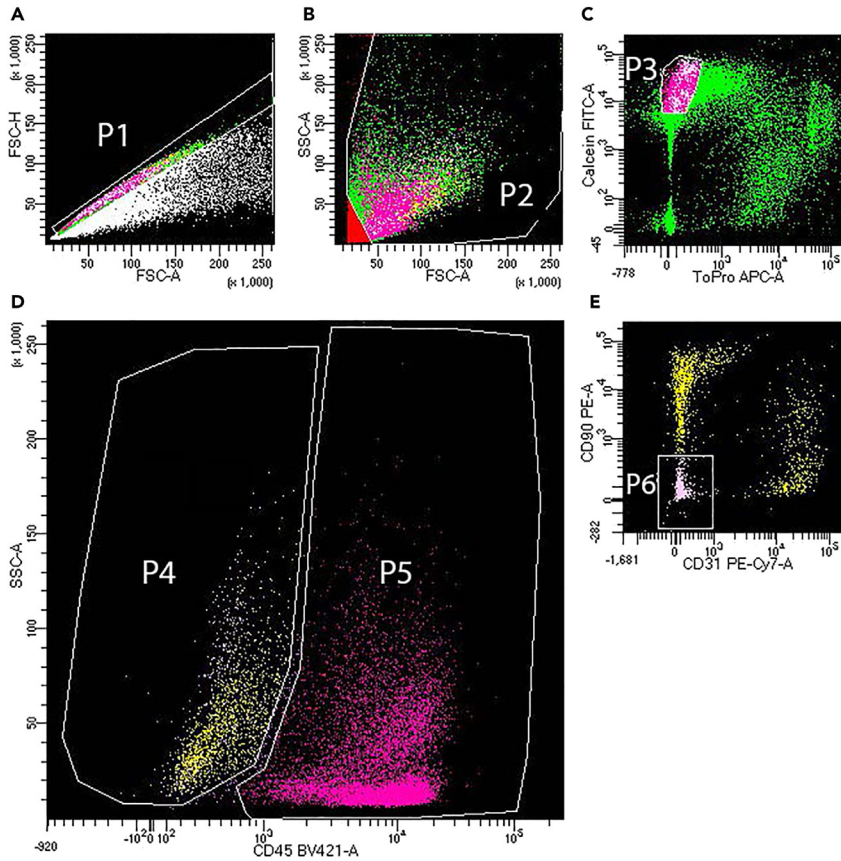


Figure 4. Flow cytometric isolation of malignant cells from an HNSCC primary tumor sample

- (A) Forward scatter height versus area criteria are used to select singlet cells (P1 gate).
 (B) Side scatter area versus forward scatter area criteria are used to eliminate debris (P2 gate).
 (C) Live cells are identified as calcein^{high} and To-Pro-3^{low} (P3 gate).
 (D) Non-immune cells are identified as CD45^{low} (P4 gate), while immune cells are identified as CD45^{high} (P5 gate).
 (E) Malignant cells are identified as CD90^{low} and CD31^{low} (P6 gate).

discard doublets (Figure 4A), side scatter area versus forward scatter area criteria to discard debris (Figure 4B), and gating on calcein^{high} and To-Pro-3^{low} cells to discard dead cells (Figure 4C). Similar criteria may be applied to HNSCC primary tumors (Figures 4A–4C), metastatic HNSCC lymph nodes (Figures 5A–5C), and ACC primary tumors (Figures 6A–6C).

Our previous studies demonstrated that HNSCC tumors exhibit high cellular heterogeneity, consisting of several cell types including malignant epithelial cells, immune cells, endothelial cells, and cancer-associated fibroblasts.¹ Given the low fraction of malignant cells in primary tumor samples, isolation of sufficient malignant cells for sequencing or cell culture applications often necessitates enrichment for malignant cells. Prior studies have utilized selection markers to actively identify malignant cell populations; such markers include CD44,^{4,5} aldehyde dehydrogenase,⁶ and EpCAM.⁷ Though widely used, active selection of malignant cells risks missing a proportion of cells that do not stain highly for a positive selection marker and we do not favor this strategy. Instead, we favor depletion of non-malignant cell types, as we believe this strategy is most likely to capture the full diversity of malignant cells. We utilize CD45, CD90, and CD31 as markers to deplete immune cells, fibroblasts, and endothelial cells, respectively (Figures 4D and 4E).^{8–10} A similar strategy can be applied to HNSCC metastatic lymph nodes, where we find even higher proportions of non-malignant cell types (Figures 5D and 5E). In contrast, ACC tumor samples tend to have higher percentages of malignant cells and, thus, CD45 staining is sufficient to exclude immune cells and enrich for

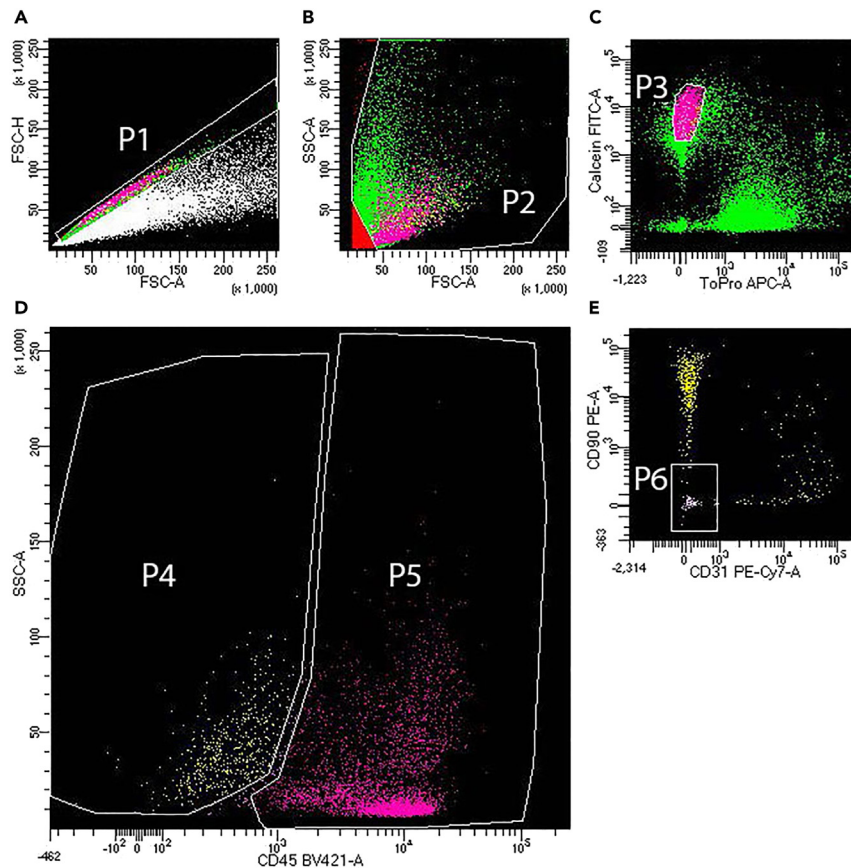


Figure 5. Flow cytometric isolation of malignant cells from an HNSCC metastatic cervical lymph node

(A) Forward scatter height versus area criteria are used to select singlet cells (P1 gate).

(B) Side scatter area versus forward scatter area criteria are used to eliminate debris (P2 gate).

(C) Live cells are identified as calcein^{high} and To-Pro-3^{low} (P3 gate).

(D) Non-immune cells are identified as CD45^{low} (P4 gate), while immune cells are identified as CD45^{high} (P5 gate). There is a lower fraction of non-immune cells in the cervical lymph node, as compared with the primary tumor (Figure 4).

(E) Malignant cells are identified as CD90^{low} and CD31^{low} (P6 gate). There is a lower fraction of CD90^{low} and CD31^{low} cells in the lymph node, as compared with the primary tumor (Figure 4).

malignant cells (Figures 6C and 6D). In these tumors, we find that depletion of fibroblasts and endothelial cells, which constitute smaller fractions of cells present, is not necessary.

LIMITATIONS

This protocol is optimized to process head and neck tumors, specifically HNSCC and ACC. There is limited evidence supporting the adaptation of this protocol to process tumors derived from other anatomical sites.

Our protocol enriches for malignant cells by depleting non-malignant cell types (e.g., immune cells, fibroblasts, endothelium) defined by cell surface markers (CD45, CD90, and CD31, respectively). Given the low fraction of malignant cells in highly heterogeneous HNSCC tumors, this approach is critical to sequencing an adequate number of malignant cells for further analysis.^{11,12} However, we do acknowledge that this approach risks losing rare malignant cells that may stain positive for these markers and thus may miss certain subpopulations of cells or create systematic differences between the distribution of sorted cells and the *in vivo* cell distribution.

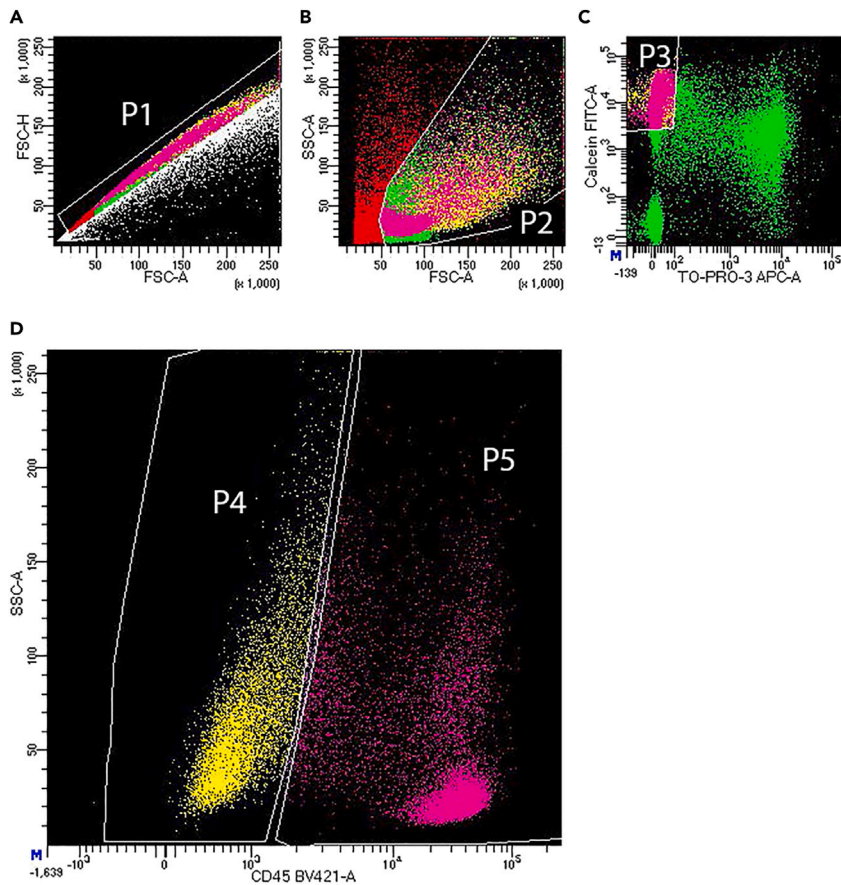


Figure 6. Flow cytometric isolation of malignant cells from an ACC primary tumor

(A) Forward scatter height versus area criteria are used to select singlet cells (P1 gate).

(B) Side scatter area versus forward scatter area criteria are used to eliminate debris (P2 gate).

(C) Live cells are identified as calcein^{high} and To-Pro-3^{low} (P3 gate).

(D) Non-immune cells are identified as CD45^{low} (P4 gate). There is a higher fraction of non-immune cells in ACC primary tumors, as compared with HNSCC primary tumors (Figure 4), thus obviating the need for additional depletion of fibroblasts and endothelial cells required with the latter.

TROUBLESHOOTING

Problem 1

Difficulty triturating tumor specimen.

Potential solution

- For larger, more difficult tumors that are challenging to triturate, consider partitioning the sample into two or more separate specimens and performing parallel dissociations.
- When triturating, ensure that there is sufficient resistance causing a small buildup of pressure as the pieces of tissue pass through the serological pipette tip. This resistive force is what drives the mechanical dissociation.
- Begin the trituration step using a larger serological pipette tip (10–25 mL) to mechanically dissociate more challenging tumor specimens.

Problem 2

Low yield of viable cells after dissociation.

Potential solution

- Prevent the excessive cell damage by limiting the maximum duration of enzymatic and manual dissociation (step 10–14) to 1 h (or less, if possible). Extended time in enzymatic mix may lead to over-digestion of the tumor sample.
- Ensure timely initiation of this dissociation and sorting protocol following the procurement of the tumor sample. If concerned for viability, use a small volume of the sample with trypan blue to perform a live cell count using a cell counter.

Problem 3

Incomplete dissociation of tumor sample.

Potential solution

- Ensure that the tumor specimen is adequately minced into 1–2 mm pieces with sterile forceps (step 1), as larger clumps of tumor tissue will incompletely dissociate and filter through the 100 μm membrane.
- At the end of the manual dissociation steps (step 10–14), add 5 μL of the sample to 5 μL of trypan blue and visualize cellular morphology on a microscope. If there are many clumps of cells and cell doublets, consider doing an additional round of trituration and incubation.
- Place residual unfiltered tumor tissue into new enzyme mix and repeat the tumor dissociation protocol beginning at step 10.

Problem 4

Weak/Diminished fluorescence intensity.

Potential solution

- Ensure that the proper amount and concentration of antibody is used.
- Ensure that the antibody has not undergone photo-oxidation; replace antibody if this is the case.
- Ensure that the lasers of the flow cytometry machine are adequately aligned.

Problem 5

Poor single-cell RNA sequencing results.

Potential solution

- To optimize cell sorting for single-cell RNA sequencing, we have found that utilizing a 100 μm nozzle, rather than a 70 μm nozzle, is critical in preventing premature cell lysis and has significantly improved the quality of our results.
- For plate-based protocols, ensure immediate sealing and snap freezing of plates with sorted cells in an RNAase free environment to prevent degradation of RNA from lysed single cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Anuraag S. Parikh (asp2145@cumc.columbia.edu) and Sid Puram (sidpuram@wustl.edu).

Materials availability

There were no newly generated materials associated with this protocol.

Data and code availability

There were no new datasets or code generated for this study.

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AUTHOR CONTRIBUTIONS

Conception and methodology, A.S.P., S.V.P.; writing, O.O., A.S.P.; reviewing and editing, V.Y., C.M., S.F., D.T.L., H.N., and S.V.P.; funding and supervision, A.S.P, S.V.P., and H.N.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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