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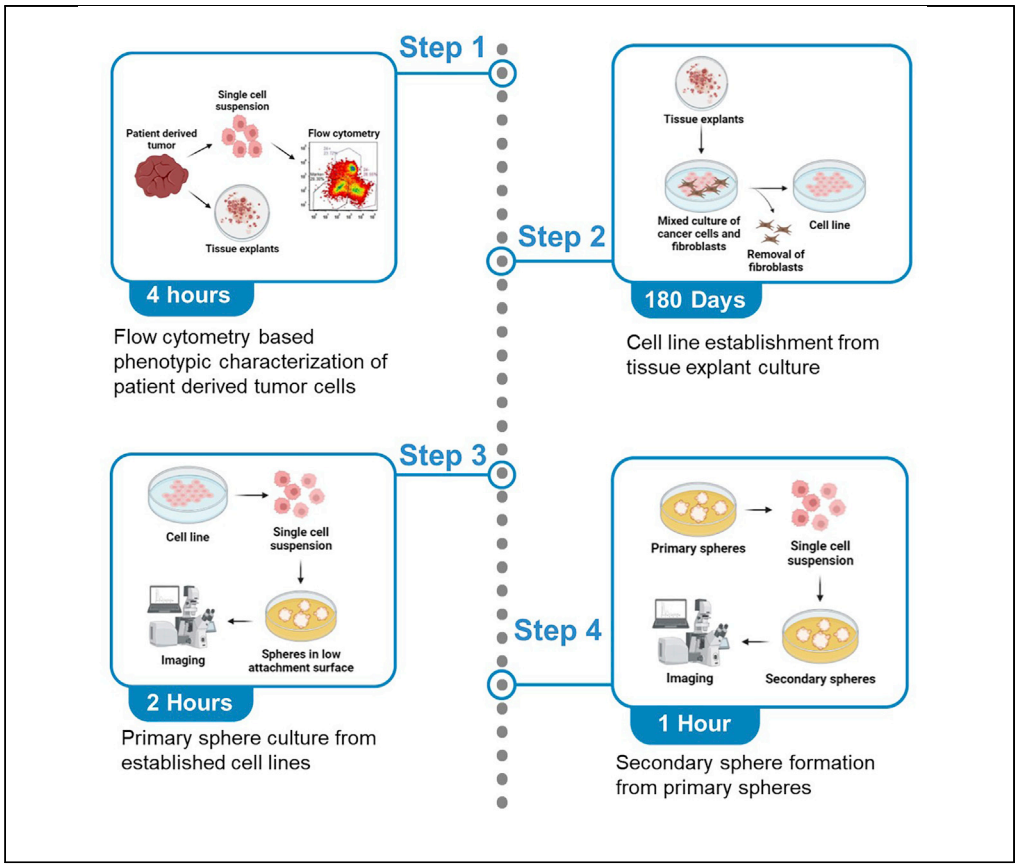
Protocol

Characterizing diversity among human oral stem-like cancer cells using flow cytometry



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

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Highlights

Enzymatic digestion protocol for surgically resected human oral tumor tissues

Flow-cytometry-based characterization of diversity in oral SLCCs

Derivation of primary cultures and cell lines from tumor explants

Protocol for growing 3D spheroid cultures of hybrid states of oral SLCCs

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Characterizing diversity among human oral stem-like cancer cells using flow cytometry

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SUMMARY

We have developed the protocol of flow cytometry for characterizing diversity among oral stem-like cancer cells (SLCCs) using CD44, CD24, and aldehyde dehydrogenase (ALDH) in oral tumors. We are also reporting the protocol for tumor-derived explant cultures to develop oral cancer cell lines and enriching these diverse hybrid states of cancer cells in 3D spheroids from established cell lines. For complete details on the use and execution of this protocol, please refer to Vipparthi et al. (2022) and Vipparthi et al. (2021).

BEFORE YOU BEGIN

Gingivobuccal complex is the most common site of oral cancer incidences in India as well as in other South Asian countries (Misra et al., 2008). Socio-cultural and environmental aspects are believed to be risk-factors for gingivobuccal complex cancers (Ram et al., 2011). Gingivobuccal oral cancers are heterogeneous and genomics studies have been increasing our understanding about the intratumoral heterogeneity of this disease (Zandberg et al., 2019). However, studying the biology of these heterogeneous subpopulations with respect to the cancer behavior, treatment responses or testing of new therapies etc., requires appropriate *ex-situ* models. Therefore, there is a necessity of a simple and convenient procedure for *ex vivo* propagation of human oral tumor samples. Our protocol explains the experimental procedures of enzymatic dissociation of human oral tumor samples, flow-cytometry based characterization of phenotypic diversity of oral cancer cells and derivation of primary cultures from tissue-explants. 3D-spheroid cultures provide insights, that are usually not possible with the traditional 2D-cultures. Spheroid cultures more reliably recapitulate the primary tumor regarding the multicellular context, cell-cell interactions, signaling response to soluble factors, oxygen and nutrient accessibility in a gradient, and mechanotransduction (Jensen and Teng, 2020). Therefore, we have standardized the protocol of 3D-spheroid generation and passaging protocol to enrich diverse states of stem-like cancer cells (SLCCs) from oral-tumor derived cell lines.

Institutional permissions

All experiments using patient-derived tissues must be performed after appropriate approvals, patients consent and in accordance with the respective institutional and national guidelines and regulations. Tissue samples were collected from Tata Medical Center, Kolkata (TMC, Kolkata) with the approval of the Institutional Review Board of the Tata Medical Center and Institutional Research Ethics Committee of National Institute of Biomedical Genomics, Kalyani (NIBMG, Kalyani). Also, all experiments were performed following institutional biosafety guidelines.

Preparation of the solutions

All required solutions can be made freshly before the experiment or may be stored at a specific temperature for some duration, as mentioned in the methods section. For details of the materials, refer to the key resources table as well as the materials and equipment section below.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421 Mouse Anti-Human CD24 (Clone: ML5) (1:20 dilution)	BD Biosciences	Cat#562789
BV786 Mouse Anti-Human CD44 (Clone: G4426) (1:20 dilution)	BD Biosciences	Cat#564942
Human Hematopoietic Lineage Antibody Cocktail, APC (1:5 dilution)	eBioscience	Cat#22-7776-72
Human BD Fc Block antibody (1:20 dilution)	BD Biosciences	Cat#564220
Anti-human CD31 APC Conjugated (1:20 dilution)	eBioscience	Cat#17-0319-41
Biological samples		
Surgically resected human oral tumor tissue	Tata Medical Center, Kolkata, India	N/A
Oral cancer cell lines and spheroids	Our own laboratory at National Institute of Biomedical Genomics (NIBMG), Kalyani, India	N/A
Chemicals, peptides, and recombinant proteins		
DMEM/F12 Media	Thermo Fisher Scientific	Cat#11330
EpiLife Media	Thermo Fisher Scientific	Cat#MEP500CA
HBSS Buffer	Thermo Fisher Scientific	Cat#14175
HEPES	Thermo Fisher Scientific	Cat#15630
ACK Lysing Buffer	Thermo Fisher Scientific	Cat#A10492-01
Trypsin-EDTA	Thermo Fisher Scientific	Cat#25300
Trypsin Neutralizer Solution	Thermo Fisher Scientific	Cat#R002100
Sodium Bicarbonate	Thermo Fisher Scientific	Cat#25080-094
B-27	Thermo Fisher Scientific	Cat#12587-010
EGF	Thermo Fisher Scientific	Cat#PHG0311
bFGF	Thermo Fisher Scientific	Cat#PHG0263
Geltrex	Thermo Fisher Scientific	Cat#A14132-02
Antibiotic-Antimycotic mix (100×)	Thermo Fisher Scientific	Cat#15240-062
Hydrocortisone	Sigma	Cat#H0888
Propidium Iodide	Sigma	Cat#P4170
Collagenase/Hyaluronidase Mix (10×)	STEMCELL Technologies	Cat#07912
DNase-I	STEMCELL Technologies	Cat#07900
Heat Inactivated FBS	Thermo Fisher Scientific	Cat#16140-071
Dimethyl Sulfoxide (DMSO)	Sigma	Cat#SHBJ5443
Rock1 Inhibitor (50 mM)	Selleckchem	Cat#S1049
DPBS (1×)	Thermo Fisher Scientific	Cat#14190144
Collagen I, Rat tail	Thermo Fisher Scientific	Cat#A1048301
β-Mercaptoethanol, Molecular biology grade	Sigma	Cat#M6250
Gentamicin (50 mg/mL)	Thermo Fisher Scientific	Cat#15750-060
Trypan Blue	Thermo Fisher Scientific	Cat#15250061
ALDEFLUOR™ kit	STEMCELL Technologies	Cat#01700
ALDEFLUOR assay buffer	STEMCELL Technologies	Cat#01702

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dispase	STEMCELL Technologies	Cat#07923
Insulin-Transferrin-Selenium (ITS) 100×	Thermo Fisher Scientific	Cat#41400-045
Experimental models: Cell lines		
Human oral cancer: GBC02 cells	NIBMG, India	Vipparthi et al. (2021)
Human oral cancer: GBC035 cells	NIBMG, India	Vipparthi et al., 2021
Software and algorithms		
Fiji	ImageJ	https://imagej.nih.gov/ij/download.html
Prism	GraphPad Software, LLC	https://www.graphpad.com/scientific-software/prism/
FCS Express Launcher	De Novo Software	https://denovosoftware.com/
Celleste 4.1 64 bit	Thermo Fisher Scientific	https://www.thermofisher.com
BioRender	BioRender	https://biorender.com/
Other		
35 mm cell culture Dish	Eppendorf	Cat#0030700112
60 mm cell culture Dish	Eppendorf	Cat#0030701119
100 mm cell culture Dish	Eppendorf	Cat#0030702115
Corning Costar ultra-low attachment microplates 6-well	Corning	Cat#3471
Corning Costar ultra-low attachment microplates 96-well	Corning	Cat#3474
Cryochill vials	Tarsons	Cat#523182
Corning 500 mL filter system	Corning	Cat#431097
Cryochill, 1 degree cooler	Tarsons	Cat#525000
Cell scraper	Tarsons	Cat#960052
Cell strainer 40 μM	Corning	Cat#431750
BD FACS Aria-Fusion	BD Biosciences	N/A
Olympus CKX41 inverted Microscope	Olympus Life Science	N/A
EVOS M7000 Imaging system	Thermo Fisher Scientific	Cat#AMF7000

MATERIALS AND EQUIPMENT

Tissue transport media		
Reagent	Final concentration	Amount
DMEM/F12K Media	N/A	41.810 mL
Gentamicin	2×	100 μL
Antibiotic-Antimycotic Mix (100×)	2×	1 mL
β-mercaptoethanol (100 mM)	100 μM	50 μL

Tissue dissociation media		
Reagent	Final concentration	Amount
DMEM/F12K Media	N/A	41.810 mL
Gentamicin	2×	100 μL
Antibiotic-Antimycotic Mix (100×)	2×	1 mL
β-mercaptoethanol (100 mM)	100 μM	50 μL
Collagenase/Hyaluronidase (10×)	1×	5 mL
DNase I (2,500 units/mL)	100 units/mL	2 mL
Rock-Inhibitor (50 mM)	40 μM	40 μL
Total	N/A	50 mL

Tissue explant culture media		
Reagent	Final concentration	Amount
Epilife Media or DMEM/F12K Media	N/A	44.210 mL
FBS	10%	5 mL
Antibiotic-Antimycotic Mix (100×)	2×	100 μL
Gentamicin	2×	100 μL
β-mercaptoethanol (100 mM)	100 μM	50 μL
ITS (100×)	1×	500 μL
Rock- Inhibitor (50 mM)	40 μM	40 μL
Total	**N/A**	**50 mL**

Complete DMEM/F12K media		
Reagent	Final concentration	Amount
DMEM/F12K Media	N/A	445 mL
FBS	10%	50 mL
Antibiotic-Antimycotic Mix (100×)	1×	5 mL
Total	**N/A**	**500 mL**

Note: Complete DMEM/F12 medium is used for culturing the GBC035 cell line.

Complete EpiLife media		
Reagent	Final concentration	Amount
EpiLife Media	N/A	474.3 mL
FBS	2%	10 mL
Antibiotic-Antimycotic Mix (100×)	1×	5 mL
Hydrocortisone	0.4 μg/mL	0.5 mL
B27	1×	10 mL
EGF	20 ng/mL	0.1 mL
bFGF	20 ng/mL	0.1 mL
Total	**N/A**	**500 mL**
Store at 4°C for a maximum of 20 days. Before using, pre-warm it to 37°C.		

Note: GBC02 cell lines are propagated in the complete EpiLife media.

Spheroid growth culture media (1× supplements)		
Reagent	Final concentration	Amount
Plain media without FBS (DMEM/F12, EpiLife, MEM)	N/A	9.686 mL
Antibiotic-Antimycotic Mix (100×)	1×	0.1 mL
B27	1×	0.2 mL
Hydrocortisone	0.4 μg/mL	10 μL
EGF	20 ng/mL	2 μL
bFGF	20 ng/mL	2 μL
Total	**N/A**	**10 mL**

Spheroid growth culture media (5× supplements)		
Reagent	Final concentration	Amount
Plain media without FBS (DMEM/F12, Epilife, MEM)	N/A	8.830 mL
Antibiotic-Antimycotic Mix (100×)	1×	0.1 mL
B27	5×	1 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Hydrocortisone	2 μg/mL	50 μL
EGF	100 ng/mL	10 μL
bFGF	100 ng/mL	10 μL
Total	N/A	10 mL

Cryopreservation media		
Reagent	Final concentration	Amount
Plain Media + Antibiotic-Antimycotic Mix (1×)	N/A	0.7 mL
FBS	20%	0.2 mL
Dimethyl Sulfoxide (DMSO)	10%	0.1 mL
Total	N/A	1 mL

⚠ CRITICAL: DMSO is toxic to cells; add it dropwise slowly to cells containing chilled media.

HBSS(+) buffer		
Reagent	Final concentration	Amount
HBSS Buffer	N/A	48.75 mL
Antibiotic-Antimycotic Mix (100×)	1×	0.5 mL
FBS	0.5%	0.25 mL
HEPES (1 M)	10 mM	0.5 mL
Total	N/A	50 mL

STEP-BY-STEP METHOD DETAILS

We generally perform this method with patient derived oral tumor tissues and oral cancer cell lines. However, this protocol may be compatible with other types of solid tumors and primary cells with simple modifications.

Collagen coating of culture dishes

Timing: 1 h

To culture the dissociated tumor tissues, we have used collagen coated cell culture surfaces. The following method is used for coating the surface of cell culture dishes with collagen-I.

1. Dilute collagen-I (stock 3 mg/mL) in PBS to 50 μg/mL for the required volume to cover the surface of the dish (for each 10 cm dishes we use 5 mL of collagen coating solution).
2. Incubate dishes at 37°C sterile incubator for 30 min.
3. Aspirate collagen solution and wash the coated surface twice with PBS.
4. Air dry the dishes/plates in the bio-safety cabinet.
   a. Seal the unused plate/dishes and store at 4°C for later use.

Tumor dissociation

Timing: 2 h

The following section will accomplish collection and transportation of tumor samples to the lab, dissociation of tumor to partially digested tissues for explant cultures as well as single cell preparation for flow-cytometry.

5. Transport samples to the lab in freshly prepared tissue transport media (prepared fresh and did not store) under chilled condition using frozen gel packs.
6. Wash tumor sample pieces with PBS containing 2× Gentamicin, 2× antibiotic-antimycotic mix (Penicillin, Streptomycin, Amphotericin B - mix) with gentle rocking for five minutes followed by centrifugation at 500 × *g* for 10 s.
7. Wash the sample with ACK lysis buffer at room-temperature (24°C–27°C) for two minutes with gentle rocking, and centrifuge at 500 × *g* for 10 s.
8. Wash twice with chilled PBS containing 2× Gentamicin, 2× antibiotic-antimycotic mix. Centrifuge at 500 × *g* for 10 s.
9. Transfer in chilled tissue transport media. Keep it on ice.
10. Put a cell culture dish on ice and transfer the sample in the dish in 500 μL tissue transport media.
    a. Mince the sample into fine pieces, using a surgical blade.

Note: This minced tissue slurry should be pipettable with 2 mL pipette.

11. Collect the samples and resuspend in 5 mL of tissue dissociation media (prepared fresh and did not store).
12. Transfer the tissue suspension in a 15 mL tube and put in a rack, placed in a 37°C water bath.
13. Digest the samples for 60 min with mixing by pipetting up and down by 2 mL serological pipette with gentle pressure against the bottom of the tube for 5 times in every 15 min.

Note: Keep monitoring the process of tissue digestion by taking a small portion of samples over the glass slide and checking under the microscope. Digesting tumor pieces should not remain bigger than 200–300 micron in size.

14. Spin at 500 × *g* for 10 s.

Note: This will allow undigested tissue samples to get sedimented in bottom of the tube and digested single cells will remain in suspension.

15. Collect all the cells in suspension in a fresh tube (Tube A).
16. Wash the pellet once by adding 1 mL tissue transport media and centrifuging at 500 × *g* for 10 s.
17. Collect all the cells in suspension and add them to 'Tube A'.

Note: This tube contains fully digested single-cell suspension of the tissue.

18. Pellet contains partially digested tumor pieces.
    a. Resuspend the tumor pieces in 1 mL of tissue transport media.
19. Transfer 250 μL of suspension with these partially digested tissue pieces to a fresh tube and label the tube as 'Tube B'.
    a. Set aside on ice for explant culture, as mentioned later.
20. Pellet the remaining partially digested tissue and add 1 mL of tissue dissociation media.
21. Incubate for 10–15 min at 37°C.
22. Pipette up and down the tissue slurry for 5 times, and check for dissociation.
23. Pellet down all indigestible tissue parts at 500 × *g* for 5 min.
24. Collect all the cells in suspension and transfer to 'Tube A'.

Processing of cell suspension (tube A) for flow cytometry

25. Pass the cell suspension through a 40 μm cell strainer, and spin at 500 × *g* for 5 min.
26. Add 200 μL of ACK lysis buffer to the pellet and gently rock the pellet for 1 min.
27. Pellet down the cells by centrifuging at 500 × *g* for 3 min. Remove buffer.

28. Wash cell pellets with freshly prepared chilled HBSS(+) buffer twice and again resuspend the cells in chilled HBSS(+) buffer.
    a. Prepared HBSS(+) buffer can be stored at 4°C for 15 days.
    b. Keep the tube on ice.
29. Count the cells with trypan blue and proceed for flow-cytometry, as mentioned below.

Flow cytometry

Timing: 2 h

The following section will accomplish the sample preparation for flow-cytometry, acquisition of data and data analysis for detection and quantification of putative oral-SLCCs and its hybrid states.

Aldefluor assay and antibody-based phenotyping

The ALDEFLUOR™ reagent is a proprietary item of STEMCELL Technologies, Vancouver, Canada. This is provided in an inactive and stable form (BODIPY- aminoacetaldehyde-diethyl acetate, BAAA-DA). The dry ALDEFLUOR™ reagent needs to be dissolved in DMSO, converted to the fluorescent-activated ALDEFLUOR™ reagent (BAAA) by treatment with 2 N HCl and diluted with ALDEFLUOR™ assay buffer as per the instructions provided by the manufacturer (link here). Aliquot the activated ALDEFLUOR reagent and store at −20°C.

Note: Always keep reagents on ice and away from light, while using.

Cell sample preparation

30. Count cells in 'Tube A' and take the required volume of cell suspension (approximately 2×10^6 cells) in a 15 mL tube after calculations.

Note: This 2×10^6 cells is the count of large cells in digested cell suspension of tumors. Very small lymphocytes are not included in this count or in further calculations. We generally have observed that contaminating lymphocytes contribute to 30%–50% of total cells.

31. Pellet down the cells (at 400 × *g* for 5 min) and resuspend in 2 mL of ALDEFLUOR™ assay buffer at the dilution of 1×10^6 cells in 1 mL of assay buffer.
32. Take two separate 1.5 mL tubes and label them as 'unstained' and 'control'.

Note: The control tube serves as a negative control for ALDEFLUOR Assay as well as negative control of antibody staining (isotype-antibody staining).

33. Add 500 μL of cell suspension in unstained and negative control tubes. Remaining 1.0 mL cell suspension tube is labeled as 'Test'.
34. In the control tube, add 5 μL of N,N-diethylaminobenzaldehyde (DEAB) (5 μL of DEAB /500 μL of cell suspension).
    a. Recap the DEAB container, immediately.

⚠ CRITICAL: Preincubate all the tubes from steps 33 and 34 at 37°C for 10 min, before advancing to further steps.

35. Next, add 2.5 μL of activated ALDEFLUOR reagent in the 'Control' tube and 5 μL in 'Test' tubes, under dark condition.

⚠ CRITICAL: Enzymatic reaction begins immediately after addition of activated substrate to the cell suspension. Thus, reagent should be added to the DEAB - control tube without any delay.

Note: Use a separate tip for dispensing reagent in each tube.

36. Mix by inverting the tubes 5 times.
37. Incubate all tubes for 1 h at 37°C in a water bath.
38. After incubation, transfer all tubes to ice.
39. Centrifuge the tubes for 5 min at 400 × *g*.
40. Discard the supernatant and resuspend cell pellets in ice cold ALDEFLUOR™ assay buffer for immunophenotyping at the dilution of 1 × 10^6 cells per 100 μL.

⚠ CRITICAL: Before proceeding to next step, perform Fc receptor blocking using Human BD Fc Block antibody as per the manufacturer's protocol (link here) to avoid non-specific false-positive binding of antibody.

41. Add APC-conjugated lineage antibody cocktail (in 1:5 dilution), APC-conjugated human CD31 antibody (in 1:20 dilution), BV421-conjugated Human CD24 antibody (in 1:20 dilution) and BV786-conjugated Human CD44 antibody (in 1:20 dilution) in the 'Test' tube. Add isotype control antibody in the DEAB-Control tube.
42. Incubate tubes for 30 min at 4°C (in the dark).
43. After incubation, wash the cells twice with the ice-cold ALDEFLUOR™ assay buffer and centrifuge at 400 × *g* for 5 min.
44. Pass cell suspension through 40 μm cell strainer and collect the flow through, to avoid any cell clump.
45. Add propidium iodide at final concentration of 2 μg/mL and incubate for 5 min.

Note: Propidium iodide is added to exclude all dead cells at the time of analysis. No need to wash propidium iodide for subsequent steps.

Note: These prepared tubes can be stored only for short period of time (1 h) at 4°C in dark until they are used for flow-cytometry analysis; otherwise analyze immediately.

Note: For cell sorting purpose, collection buffer may be made by adding 5% FBS in HBSS (+) tubes and stored at 4°C, beforehand. Collect sorted cells into appropriately labeled tubes containing collection buffer.

Phenotype analysis of CD24, CD44, and ALDH-high cells by BD FACS Aria-Fusion flow cytometer

46. Set the gates using the strategy as shown below in Figure 1A.
47. Set forward scatter (FSC) and side scatter (SSC) dot plot for excluding debris and clumped cells (Figures 1B-i, ii, iii).
48. Use unstained cells to set the appropriate voltages for FSC, SSC, and viability (propidium iodide fluorescence) parameters (Figure 1B-iv).
49. Use DEAB-'control' to set the appropriate voltages for background fluorescence parameters for APC, BV421, BV786, and Aldefluor fluorescence (ALDH) (Figures 1C- i, ii, iii, respectively).
50. Quantify cell populations (Figures 1D- i, ii, iii, iv).

Note: For flow-cytometry-based phenotyping, configuration of the instrument must be checked. The combination of fluorochrome used by us did not require any compensation to be performed. Therefore, this combination allows you to avoid compensation controls.

Establishment of primary cultures from tissue explants (TUBE B)

Timing: 12–20 weeks

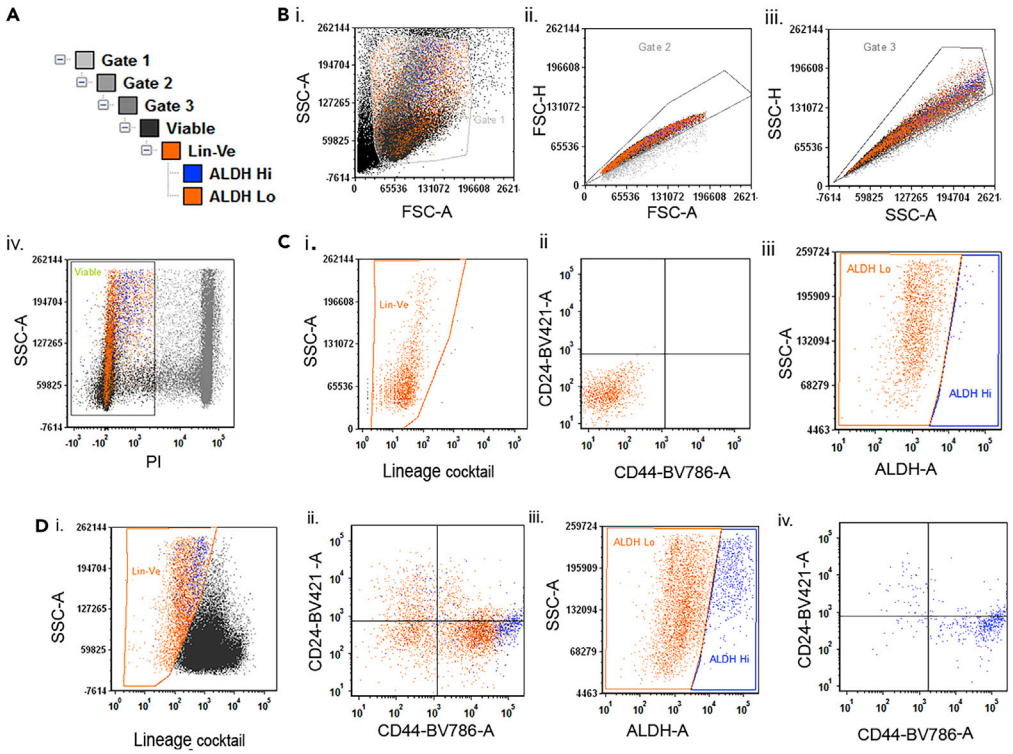

Figure 1. CD24, CD44, and ALDH expression in oral tumors

(A) Gating strategy has been shown.

(B) (i) Gate 1 shows all intact cells with high-FSC and SCC parameters. Cell debris with low-FSC and SCC as well as aggregates/ large cells with very high FSC and/or SCC values were excluded. B (ii, iii) Gate 2 and 3 were made to include only singlet cell population. B (iv) Live cells were included for analysis by drawing a viable gate for propidium iodide negative population.

(C) Using DEAB-control and isotype control antibody, gates for lineage negative as in C (i), CD24/CD44 negative as in C (ii) and ALDH-Low as in C (iii) population is shown.

(D) APC-conjugated lineage antibody cocktail mixed with CD31-APC was used to separate lineage marker-positive and CD31-positive cells. Lineage antibody-negative gate was drawn for including and analyzing lineage marker and CD31-negative tumor cells (D-i). From this gate, cells populated in respective gates are shown for CD24/CD44 expression (D-ii), ALHD-High and Low (D-iii) and CD24/CD44 expressing cells with ALDH-High phenotype (D-iv).

The following section describes the method for development of pure cultures of cancer cells along with elimination of contaminating fibroblasts growth in primary explant cultures from partially digested oral tumor tissue explants.

51. Place a few partially digested tissue-explants in 20–30 μL volume as droplets over collagen-coated cell culture plates in tissue explant culture media.
    a. Media may be stored at 4°C for maximum of 15 days after preparation.
52. It takes a few hours for tissue to get attached to the coated surface. Make sure that the media is not dried-up. Monitoring every hour under microscope is necessary.
53. After attachment, add extra media just enough to cover the whole surface.

Note: An outgrowth of cells can be started from the attached tissue explants. The originated cells are mostly spindle-shaped fibroblast and other irregular or star-shaped epithelial cells.

54. Media should be refreshed twice a week.
55. Once the outgrowth of cells reaches confluency, passage the cells.

56. For passaging of the cells, aspirate the media gently without disturbing the attached tissue explant. Wash twice with PBS.
57. Add 1 mL, 37°C pre-warmed trypsin.
    a. Keep monitoring cell detachment under the microscope.
    b. Add serum-containing media to neutralize trypsin.

Note: Due to this partial trypsinization, the fibroblast cells come off the plate earlier than the growing epithelial cells.

58. Collect the detached cells and grow separately in a fresh well of multi-well plate.
59. Add fresh tissue explant culture media in the plate containing explants and allow it to grow.
    a. Repeat steps 56–59 of partial trypsinization to collect all outgrowing cells from the tissue explants unless the tissue is exhausted or detached, in 4–8 weeks.
60. To enrich epithelial cells, keep repeating partial trypsinization until the epithelial cells outgrow and become major population in the plate.
    a. This may take 8–12 weeks.

Note: Outgrowing cells from tissue explants are initially mixed cultures of epithelial cells and fibroblasts. Presence of these fibroblasts is beneficial for the epithelial cells to survive and adapt.

Alternatives: Enriching epithelial cells can be maintained separately in complete DMEM-F12K medium or in complete EpiLife medium with low serum.

Note: These enriched epithelial cells are ready to be cryopreserved and sub-culturing as cell lines. Please see the critical points and notes below.

⚠ CRITICAL: During cell culture establishment, there is a major threat of microbial contamination present in the oral cavity of the patient tumor tissues. Thus, washing with antibiotic mix is the most critical step. Therefore, tissue explants are initially grown with a higher dose of antibiotic-antimycotic mix.

⚠ CRITICAL: Removal of fibroblasts from the mixed culture after partial trypsinization is often associated with stress in cancer cells and loss of growth (Figure 2). Therefore, it is critical to monitor cell morphology and symptoms of stress (including vacuolization, floating cells) in growing epithelial cells. Re-introduction of fibroblasts or keeping the mixed culture for a longer period of time may be required under this situation. Also, check the troubleshooting section for other important points.

Note: Once the cell line has been established, the STR profiling can be done on a specific interval to authenticate the origin of the cell lines. Purity of the established cell cultures can be characterized by flow-cytometry using marker for epithelial cells ($EpCAM^+$) and fibroblasts ($CD90^+$) or any other assay dependent markers.

Cell culture and passaging

Timing: 5 days

The following section will accomplish the maintenance of established cell lines in cultures and its cryopreservation for future usage.

61. Cell Culture.
    a. Transfer the cryovials containing frozen cells from liquid nitrogen to a 37°C water bath.

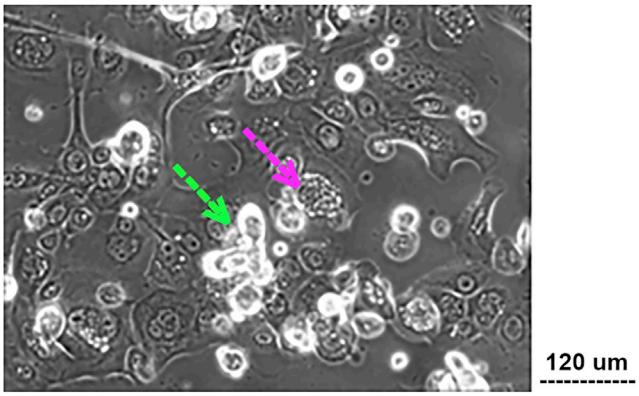

Figure 2. Removal of fibroblasts from growing mixed cultures results in vacuolization in cancer cells
Pink arrow pointing a cell having multiple vacuoles and green arrow pointing on floating dead cells.

b. Once thawed, transfer cells gently to 10 mL of plain medium in a 15 mL tube to dilute DMSO.
c. Centrifuge at 400 × *g* for 3 min.
d. Discard supernatant and resuspend in 1 mL of prewarmed complete media.
e. Pre-lay 2 mL of pre-warmed complete media into a 60 mm dish and transfer cells in a 60 mm dish having 2 mL prewarmed complete media.
   i. Any complete cell culture media (complete DMEM/F12K or EpiLife media) may be stored at 4°C for maximum of 15 days after preparation.
f. Place the plate in a 37°C incubator with 5% CO_2.

62. Sub-culturing.
   a. After 48 h of cell plating, remove media and rinse adhered cells with 2 mL of PBS to remove excess media and FBS.

Note: This process can be repeated twice, in case of strongly adherent cell lines.

   b. Add 500 µL of room-temperature (24°C–27°C) 0.05% Trypsin-EDTA, and incubate for 5–6 min in the incubator at 37°C.
   c. Add 1 mL of Trypsin neutralizer to the detached cell suspension.
   d. Collect cells in a tube and spin at 400 × *g* for 3 min at room-temperature (24°C–27°C).
   e. Discard the supernatant and resuspend the cell pellet in 2 mL of complete media.
   f. Count cells and plate at desired cell density for further usage.

Note: We prefer to revive cells in 60 mm dishes. Though for expansion, cells can be seeded in a bigger surface dish or flask. For sub-cultures we regularly seed 3 × 10^5–4 × 10^5 cells in a 60 mm dish. Cell culture medium is stored at 4°C for a maximum of 45 days. Media is pre-warmed to 37°C before using.

⚠ CRITICAL: Dead cells should be removed from the culture. If observed in higher number, PBS wash may be given once before adding fresh media. 3% sodium bicarbonate may be added in the media for fast proliferating cells to have better buffering ability of media for pH changes.

63. Cryopreservation.
   a. Resuspend 1 × 10^6 cells in 1 mL of freshly prepared chilled cryopreservation media.
   b. Cryopreservation media may be stored at 4°C for maximum of 15 days after preparation.
   c. Transfer cells-containing media in a cryovial.
   d. Place cryovial in a Cryochill 1 degree cooler and keep it at −80°C for 1 day.

e. Transfer frozen cell cryovials into liquid nitrogen.

Primary spheroid formation from patient-derived cell lines

Timing: 1 h

The following section will accomplish the growth of 3D-spheroids from established cell lines.

64. Trypsinize cells as described in the previous section, and make single cell suspension in complete media.
65. Suspend cell suspension at 1 × 10^5 cells per mL in plain media without FBS. Keep cell suspension on ice.
66. Take required cells to make cell suspension of 3,000–5,000 cells per mL in spheroid growth culture media containing 1× growth factor supplementation.

Note: Try to make spheroid growth culture media and 5× growth factor supplements, freshly before use. Can be stored up to 2 weeks at 4°C.

67. Mix ice thawed Geltrex at final concentration of 1.25% to cell suspension followed by vortexing for 5–10 s.

⚠ CRITICAL: Geltrex is a commercially available basement membrane matrix. It is aliquoted and stored at −20°C and forms a gel at room-temperature (24°C–27°C) or higher. Therefore, it requires thawing on ice. Complete thawing is crucial, and it takes at least 2 h before being ready to be used. Use chilled tips to pipette Geltrex.

68. Transfer cells in desired volume in multi-well ultra-low attachment (ULA) culture plates.
    a. 1.5 mL of cell suspension may be transferred in one well of a 6 well ULA plate.
69. Seeded plates are transferred in a 5% CO_2 incubator at 37°C. Spheroids are formed in 7–10 days of cell plating.
70. Every alternate day of cell seeding add 200 μL of 5× growth factor supplements per mL of culture media as supplement.

Alternatives: Corning Matrigel (Merck, Cat. No. CLS356234) may also be used as an alternative to Geltrex.

Passaging of primary spheroids and generation of secondary spheroid

Timing: 2 h

The following section will accomplish the passaging of 3D-spheroids forming cells and generating secondary spheroids.

71. Take primary spheroids into a fresh tube and spin at 300 × *g* for 4 min.
72. Discard supernatant and wash twice with PBS by centrifugation to remove Geltrex.
73. Add 300 μL 0.05% Trypsin-EDTA solution to spheroids and keep in the water bath maintained at 37°C for 10–15 min with intermittent vortexing.
74. Check under the microscope for complete dissociation to single cell suspension.
75. Add trypsin neutralizer solution and spin at 500 × *g* for 4 min.
76. Resuspend cell pellet in spheroid growth culture medium with 1× growth factor supplements.
77. Repeat the steps of primary spheroid formation (step 73–77) for the secondary spheroid formation.

EXPECTED OUTCOMES

The protocol described here provides a reliable method for enzymatic dissociation of human oral tumor tissues, followed by flow-cytometry analysis. Our protocol is advancing from the previously described method where oral mucosal tissues were dissociated into single cell suspension, however flow-cytometry using multiple markers were not described (Greenwell-Wild et al., 2021). Also, we have provided the detailed protocol for establishment of primary cultures of oral cancer cells from partially digested tissue explants. The flow-cytometry based analyzed cells can be sorted and subjected to several molecular assays like gene expression analysis including single cell assay. Further, in our experience, tumor explants are expected to have better success in establishing in primary cultures, as compared to completely dissociated samples for human oral tumor. We believe that our protocol can be adapted for all those tumor-types where it has been challenging to establish tumor-derived 3D-cultures directly from patient tissue-samples. The explant cultures from partially digested samples result in a self-supported system of mixed-cultures of cancer cells and fibroblasts which assist the growth of cancer cells in cultures. Next, CD44-high cells are expected to enrich oral cancer cells with stem cell-like properties. With the differential expression of CD24 and aldehyde dehydrogenase activity, we could identify differentiating subpopulations in CD44-high cells, having hybrid states of gene expression and stem cell-like activity. For more information, see the previous published study by Vipparthi et al., 2022.

LIMITATIONS

The success of this protocol depends on quality of tumor tissue samples. Possibility of getting a good number of initial explants depends on histologic and necrotic state of resected and collected tumor. In this protocol, we have limitations of not providing solutions for developing 3D-organoid cultures directly from patient samples or from single cell suspensions of the tissue derived cells. This could be due to sudden loss of supporting fibroblasts and cell-matrix attachments which are maintained in explant within the solid-tissues, for longer. Also, flow-cytometry-based downstream approach is an essential part of this protocol which cannot be replaced with other techniques. Therefore, the described method is restricted to the lab set-up equipped with a flow cytometer, which can be a limitation. The method and assays also require a certain level of expertise to successfully establish cell lines and develop 3D-spheroid cultures.

TROUBLESHOOTING

Problem 1

Clump formation during enzymatic digestion of tissues.

Tumor samples are highly heterogeneous with sample-to-sample variations with respect to cell death and necrosis. DNA released from dead cells create meshwork, which traps the tissue. This makes the whole sample very viscous and hard to digest.

Potential solution

Tissue dissociation is performed with the enzyme mix with DNase I, which will digest DNA meshwork released from dying cells. Extra DNase I can be added in the dissociation solution during incubation or whole dissociation buffer can be refreshed with additional DNase I.

Problem 2

Low frequency of lineage negative population.

As shown in Figure 1, lineage-positive cells may be present at higher frequency, depending on immune infiltration status of the tumor. Therefore, it would be difficult to estimate the frequency of diverse phenotypes of lineage-negative cancer cells.

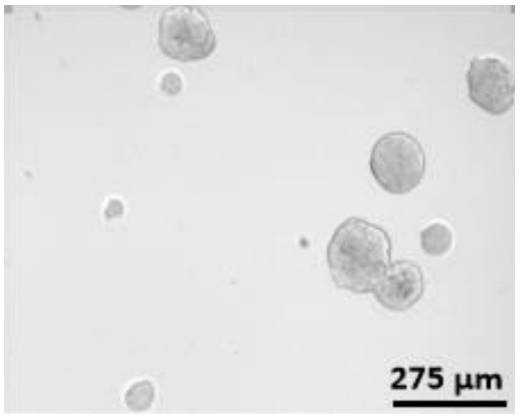

Figure 3. 3D-spheroid culture of OSCC cells and formation of fused spheroids

Potential solution

To ensure that you have enough cells to estimate frequency of cancer cells, acquire a minimum of 10,000 cells in the lineage-negative gate of flow-cytometry-plot, shown in Figure 1.

Problem 3

No growth or growth of mainly fibroblasts from tissue explants.

In our experience, we could not grow cancer cells when the tissue was completely dissociated to single cells or not digested by enzymes. Therefore, we developed this protocol where partially digested tissues were able to result in outgrowth of cancer cells. However, many tumor tissues resulted in growth of only fibroblasts. Since, initially fibroblasts grow faster and cover the surface of the culture-dishes, it is necessary to partially trypsinize the cultures and remove fibroblasts as mentioned in the protocol.

Potential solution

Partially digested tumor pieces should not be bigger than 200–300 micron in size. Larger pieces will result in no growth, or mainly fibroblasts culture. Those explants, where epithelial cells are not growing, you may change the growth media to EpiLife with growth supplements and low serum (2% FBS). This will curtail the outgrowth of fibroblasts and support epithelial cell growth.

Problem 4

Cellular aggregates or clump-formation of spheroids.

The spheroids in 3D-cultures tend to fuse and generate bigger spheroids (Figure 3).

Potential solution

Ensure to start with a single cell suspension. Keep the low cell density at the time of plating for spheroid culture (approximately 3,000–5,000 cells/ mL) based on spheroid forming ability of the cell lines. Gentle pipetting should be done after adding 5× growth factor supplements on every alternate day. We also advise to increase Geltrex concentration, if needed. This will make the matrix more viscous and harder for spheroids to move towards each other.

Problem 5

Spheroids do not dissociate into single cells after incubation at 37°C water bath.

Potential solution

For better trypsin accessibility, residual Geltrex can be digested by adding dispase at 37°C water bath for 5 min. Centrifuge the tubes to pellet spheroids and discard dispase-treated Geltrex solution

carefully with 200 μL pipette tips. Now fresh trypsin can be added onto the spheroids and incubate at 37°C in water bath, as mentioned above.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Sandeep Singh (ss5@nibmg.ac.in).

Materials availability

This study did not generate any new unique reagent.

Data and code availability

This study did not generate/analyze any dataset.

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AUTHOR CONTRIBUTIONS

S.G., K.V., and S.S. conceptualized the protocol. S.G., P.M., U.S., and K.V. performed the investigation and formal analysis. S.G., U.S., and P.M. wrote the original draft of the manuscript. S.S. acquired funding, supervised the research, and reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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