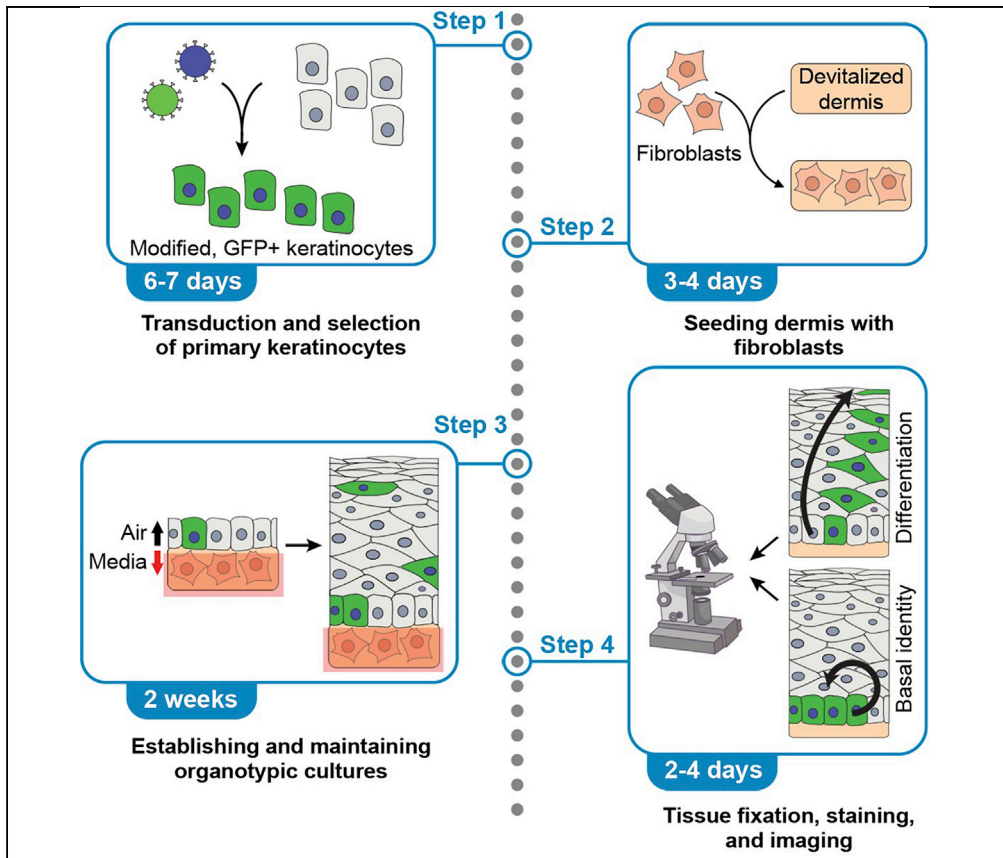


Protocol

Monitoring cell fate in 3D organotypic human squamous epithelial cultures



Here, we provide a protocol to model the effects of changes to a small number of cells, such as those arising from a mutation or a virus infection, in stratified epithelia. We describe steps for diluting engineered human keratinocytes into a larger population of unmodified cells and using these cells to grow three-dimensional organotypic cultures. We detail steps to observe effects that are not apparent in homogenous organotypic epithelial cultures by visualizing the localization of modified keratinocytes in epithelial layers.

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

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Highlights

Modify human keratinocytes to model virus infection or mutation

Generate three-dimensional organotypic cultures with a small number of modified cells

Monitor localization of modified cells in tissue

Determine the effect of virus infection or mutation on epithelial cell identity

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Protocol

Monitoring cell fate in 3D organotypic human squamous epithelial cultures

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SUMMARY

Here, we provide a protocol to model the effects of changes to a small number of cells, such as those arising from a mutation or a virus infection, in stratified epithelia. We describe steps for diluting engineered human keratinocytes into a larger population of unmodified cells and using these cells to grow three-dimensional organotypic cultures. We detail steps to observe effects that are not apparent in homogenous organotypic epithelial cultures by visualizing the localization of modified keratinocytes in epithelial layers.

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

BEFORE YOU BEGIN

Stratified squamous epithelia are multilayered tissues in which keratinocytes in the proliferative basal layer give rise to all of the upper epithelial layers. Human skin, oral mucosa, and anogenital mucosa are all stratified squamous epithelia. Three-dimensional organotypic epithelial cultures provide cell-based *in vitro* air liquid interface models that recapitulate many of the physiological aspects of the stratified squamous epithelium.^{2–5} The organotypic epithelial model used in this protocol incorporates a physiological basement membrane, primary human fibroblasts in the dermis, and primary human keratinocytes that form basal and suprabasal layers. The suprabasal layers include spinous and corneal cells.

Cell fate decisions that determine whether a keratinocyte is maintained as a basal cell or differentiates into a suprabasal cell are of interest to researchers in stem cell biology, cancer biology, and papillomavirus biology. This protocol describes a method of monitoring cell fate in organotypic epithelial cultures to determine whether the human papillomavirus (HPV) E7 oncoprotein expressed from a retroviral vector alters keratinocyte migration in organotypic culture. We have also used this protocol to monitor how CRISPR/Cas9-mediated gene deletion or doxycycline-induced gene expression impact cell fate decisions in organotypic epithelial cultures.¹ There are four major steps in the protocol: 1) transduction and selection of primary keratinocytes, 2) seeding dermis with fibroblasts, 3) establishing and maintaining organotypic epithelial cultures, and 4) fixation of organotypic epithelial cultures, staining, and imaging. In the protocol, early passage primary human foreskin keratinocytes (HFK) are used to generate passage-matched GFP-labeled and unmodified parental cells from which the user will generate organotypic epithelial cultures for cell fate monitoring.



Institutional permissions

All work with primary cells derived from human tissues was carried out with approval from the University of Pennsylvania Institutional Review Board provided through the University of Pennsylvania Skin Biology and Disease Resource-based Center (SBDRC). It is important that proper institutional and ethical approval is obtained prior to performing research involving primary cells or human tissues.

All work with recombinant DNA to generate retroviral and lentiviral vectors was carried out with approval from the University of Pennsylvania Institutional Biosafety Committee. Prior to working with retroviral and lentiviral vectors, it is important to obtain permission from the relevant bodies that regulate biosafety concerns at each institution.

Preparing devitalized dermis

⌚ Timing: 7 days

1. Prepare dermis according to the protocol in Li et al.⁵

Note: We obtained the dermis from the University of Pennsylvania SBDRC.

2. Aliquot and freeze devitalized dermis.
 - a. Using scalpel and sterile forceps, cut sheets of devitalized dermis into pieces large enough to generate 9–12 organotypic cultures.
 - b. Place pieces of devitalized dermis into 15 mL conical tubes with 5 mL of PBS plus penicillin and streptomycin.
 - c. Store pieces of devitalized dermis at -80°C for up to 4 years.

Print and sterilize 3D-printed autoclavable raft culture stands

⌚ Timing: 2–3 days

3. 3D print organotypic culture stands.
 - a. 3D print organotypic culture stands using high temperature resin.
 - b. Files for 3D printing organotypic culture stands are in [Data S1](#) and [S2](#).
4. Equilibrate newly printed stands.
 - a. Wash newly printed organotypic culture stands three times with Milli-Q water.
 - b. Incubate stands in excess PBS (around 1 L of PBS for up to 30 stands) at 37°C for 2 days. Refresh the PBS two times during the incubation.

Note: Freshly printed high temperature resin organotypic culture stands can impair growth of organotypic epithelial cultures. However, stands equilibrated in PBS before use are suitable for culturing.

5. Wash and sterilize organotypic culture stands.
 - a. Wash organotypic stands three times with Milli-Q water.
 - b. Wash organotypic stands once with 70% ethanol. Discard ethanol.
 - c. Autoclave organotypic culture stands on a liquid cycle (121°C for 30 min).

Note: For an alternative method of stand construction using tissue culture plates, please see Li et al.⁵

Add mark counting quantification toolset to Fiji

⌚ Timing: 5 min

6. Download “Mark Counting Quantification.ijm” ([Data S3](#)). Code is also contained in text boxes in “[quantification and statistical analysis](#)” section.
7. Within the folder containing your FIJI application, save “Mark Counting Quantification.ijm” in the folder: FIJI.app > macros > toolsets.
8. The toolset can be loaded in FIJI by clicking “>>” in the FIJI toolbar, and selecting “Mark Counting Quantification” in the dropdown menu.

Prepare media and solutions

⌚ Timing: 3–4 h

9. Prepare solutions listed in [materials and equipment](#) section.
10. Prepare E medium as follows⁶:
 - a. Make 10× DMEM by resuspending a 1 L size packet of powdered media in 100 mL MilliQ water.
 - b. Separately, make 10× F12 by resuspending a 1 L size packet of powdered media in 100 mL MilliQ water.
 - c. Separately, dissolve sodium bicarbonate powder in 25 mL of MilliQ water.
 - d. Separately, add 750 mL of MilliQ water to a flask.
 - e. Add the first seven components listed in the table, including the sodium bicarbonate solution, to the MilliQ water.
 - f. Adjust the pH to 7.1.
 - g. Add the FBS and bring to 1 L final volume using Milli-Q water.
 - h. Filter with 0.22 μm filter and store in two 500 mL bottles at 4°C.
 - i. Add 2.5 mL of 200× EGF to 500 mL media (5 ng/mL final concentration) before using.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IVL (mouse monoclonal) (1:100)	Santa Cruz Biotechnology	sc-398952
Anti-GFP (rabbit polyclonal) (1:2000)	Invitrogen	A6455, RRID: AB_221570
Anti-mouse IgG Alexa Fluor 488 (goat polyclonal) (1:250)	Invitrogen	A11001, RRID: AB_2534069
Anti-rabbit IgG Alexa Fluor 594 (goat polyclonal) (1:250)	Invitrogen	A11012, RRID: AB_2534079
Biological samples		
Human dermis tissues	UPenn SBDRC	N/A
Experimental models: Cell lines		
Lenti-X 293T Cells	Takara	632180
Human foreskin keratinocytes (human male, neonatal)	UPenn SBDRC	N/A
Human foreskin fibroblasts (human male, neonatal)	UPenn SBDRC	N/A
Chemicals, peptides, and recombinant proteins		
Hydrocortisone	MilliporeSigma	H-0888
Cholera toxin	MilliporeSigma	227036
Adenine	MilliporeSigma	A2786
Insulin	MilliporeSigma	I5500
Transferrin	MilliporeSigma	T1147
3,3',5-Triiodo-L-thyronine (T3)	MilliporeSigma	T6397
EGF	MilliporeSigma	324831
G418	Thermo Fisher	NC9132228
Puromycin	Thomas Scientific	C791P83
Matrigel	Corning	354234
Dulbecco's Modified Eagle Medium	Thermo Fisher	11995065

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal bovine serum (FBS)	MilliporeSigma	F2442-500ML
Keratinocyte SFM (1×) Kit (Kit includes Bovine Pituitary Extract and EGF used in Monolayer Keratinocyte Serum-Free Medium)	Thermo Fisher	17005042
Media 154	Thermo Fisher	M154500
Human keratinocyte growth supplement	Thermo Fisher	S0015
DMEM, powder	Thermo Fisher	12100046
Ham's F-12 Nutrient Mix, powder	Thermo Fisher	21700075
Penicillin/Streptomycin (100×)	Thermo Fisher	15140122
Antibiotic-Antimycotic (100×)	Thermo Fisher	15240062
Opti-MEM	Thermo Fisher	31985070
TransIT-293	Mirus	MIR 2700
Trypsin-EDTA (0.05%)	Thermo Fisher	25300054
Normal goat serum	Jackson Immunoresearch, Inc	005-000-121
Neutral buffered formalin	Azer Scientific	PFNBF-40
TWEEN-20	Thermo Fisher	J20605.AP
Triton X-100	MilliporeSigma	X100
Sodium bicarbonate	MilliporeSigma	S5761
1 M HEPES	Thermo Fisher	15630080
Tri-sodium citrate (dihydrate)	MilliporeSigma	W302600-1KG-K
Polybrene (hexadimethrine bromide)	MilliporeSigma	107689
Recombinant DNA		
p8208 MSCV-Neo C-HA EMPTY	Addgene	#163310
p6993 MSCV-Neo C-HA 18E7	Addgene	#163311
PHAGE-P-CMVt N-HA GFP	Galligan et al. ⁷	N/A
psPAX2	Addgene	#12260
pMD2.G	Addgene	#12259
pUMVC	Addgene	#8449
pCMV-VSV-G	Addgene	#8454
Software and algorithms		
(Fiji Is Just) ImageJ, Version 2.9.0	Schindelin et al. ⁸	Version 2.9.0
Other		
Scalpel handle	Oxford Instruments	51-1625-0563
#10 Scalpel blades	Thermo Fisher	17654456
Tweezers/forceps	Oxford Instruments	51-1625-0246
3D Printer, e.g., Formlabs Form 2 or Form 3	Formlabs	PKG-F3-WSVC-BASIC
High temperature resin	Formlabs	FLHTAM02
500 mL 0.22 μM filter unit	MedSupply Partners	41-FS22-500J
15 cm tissue culture coated dishes	MedSupply Partners	TL-TR4003
6 cm tissue culture coated dishes	MedSupply Partners	GR-628160
6 well tissue culture coated plates	MedSupply Partners	GR-657160
Polypropylene coplin jar	Thermo Fisher	22-038-492
PAP (liquid blocker) pen	Thermo Fisher	NC9827128
Coverslips, 24 × 50 mm, #1	Mercedes Scientific	TNR 2450
SlowFade Diamond mounting medium	Thermo Fisher	S36963
Inverted fluorescent microscope	Nikon	Eclipse Ti2

MATERIALS AND EQUIPMENT

10× Phosphate-Buffered Saline

Reagent	Amount	Final concentration
Potassium chloride (KCl)	2 g	26.8 mM (10×)
Potassium dihydrogen phosphate (KH ₂ PO ₄)	2 g	14.69 mM (10×)

(Continued on next page)

Continued

Reagent	Amount	Final concentration
Sodium chloride (NaCl)	80 g	1.36 M (10×)
Disodium hydrogen phosphate (Na ₂ HPO ₄)	11.5 g	81.01 mM (10×)
Water	To 1 L	
Total	1 L	

Autoclave and store at room temperature (approximately 20°C) for one year.

1 × Phosphate-Buffered Saline (PBS)

Reagent	Amount
10× Phosphate-Buffered Saline	100 mL
Water	900 mL
Total	1 L

Final concentration is 2.68 mM KCl, 1.47 mM KH₂PO₄, 136 mM NaCl, 8.1 mM Na₂HPO₄. pH to 7.2, adjust volume to 1 L, autoclave, and store at room temperature for one year.

293T Cell Medium

Reagent	Amount	Final concentration
DMEM	500 mL	
FBS	25 mL	5%
Penicillin/Streptomycin (100×)	5 mL	100 U/mL penicillin, 100 µg/mL streptomycin
Total	530 mL	

Store at 4°C for up to three months.

293T Cell Transfection Medium (no Pen/Strep)

Reagent	Amount	Final concentration
DMEM	500 mL	
FBS	25 mL	5%
Total	525 mL	

Store at 4°C for up to three months.

Human Foreskin Fibroblast (HFF) Medium

Reagent	Amount	Final concentration
DMEM	500 mL	
FBS	50 mL	10%
Antibiotic/Antimycotic (100×)	5 mL	100 U/mL penicillin, 100 µg/mL streptomycin, 250 ng/mL Amphotericin B
Total	530 mL	

Store at 4°C for up to three months.

Monolayer Keratinocyte Serum Free Medium

Reagent	Amount	Final concentration
Keratinocyte SFM (K-SFM)	500 mL	0.5×
Media 154	500 mL	0.5×
EGF (Supplied with K-SFM)	2.5 µg	2.5 ng/mL
Bovine pituitary extract (Supplied with K-SFM)	25 mg	25 µg/mL
Human keratinocyte growth supplement	5 mL	Proprietary (Invitrogen)
Total	1 L	

Filter sterilize using 0.22 µM filter unit and store at 4°C for up to three months.

100× E Medium Cocktail

Reagent	Amount	Final concentration
Adenine (0.18 M)	20 mL	18 mM
Insulin (5 mg/mL)	20 mL	0.5 mg/mL
Transferrin (5 mg/mL)	20 mL	0.5 mg/mL
T3 (20 nM)	20 mL	2 nM
PBS	120 mL	
Total	200 mL	

Filter sterilize using 0.22 μM filter unit and store 10 mL aliquots at –20°C for two years.

Hydrocortisone (1000×)

Reagent	Amount	Final concentration
Hydrocortisone (5 mg/mL)	1.92 mL	400 ng/mL
1 M HEPES	22.08 mL	0.92 M
Total	24 mL	

Store 1 mL aliquots at –20°C for one year.

Cholera Toxin (1000×)

Reagent	Amount	Final concentration
Cholera Toxin	1 mg	10 μg/mL
Milli-Q Water	100 mL	
Total	100 mL	

Store 1 mL aliquots at 4°C for one year.

EGF (200×)

Reagent	Amount	Final concentration
EGF	200 μg	1 μg/mL
BSA	20 mg	100 μg/mL
Milli-Q Water	200 mL	
Total	200 mL	

Store 5 mL aliquots at –20°C for one year.

E Medium

Reagent	Amount	Final concentration
DMEM (10×)	75 mL	0.75×
F12 (10×)	25 mL	0.25×
E Medium Cocktail (100×)	10 mL	1×
Penicillin/Streptomycin (100×)	10 mL	100 U/mL penicillin, 100 μg/mL streptomycin
Sodium Bicarbonate	3.06 g	36.4 mM
Hydrocortisone (1000×)	1 mL	400 μg/mL
Cholera Toxin (1000×)	1 mL	10 pg/mL
FBS	50 mL	5%
Milli-Q Water	~800 mL	
EGF (200×)	5 mL	5 ng/mL
Total	1 L	

Filter sterilize using 0.22 μM filter unit and store at 4°C for up to three months.

△ CRITICAL: Add 2.5 mL of 200× EGF to 500 mL media (5 ng/mL final concentration) before using.

Sodium Citrate Antigen Retrieval Buffer

Reagent	Amount	Final concentration
Tri-sodium citrate (dihydrate)	2.94 g	10 mM
Distilled Water	1,000 mL	
TWEEN 20	0.5 mL	0.05%
Total	1 L	

Add Tri-sodium citrate (dihydrate) to water and dissolve completely. Adjust pH to 6.0 with HCl, then add TWEEN-20 and mix well. Store at room temperature for one year.

0.05% TWEEN 20 in PBS

Reagent	Amount	Final concentration
PBS	1,000 mL	
TWEEN 20	0.5 mL	0.05%
Total	1 L	

Store at room temperature for two years.

Histology Blocking Buffer

Reagent	Amount	Final concentration
PBS	50 mL	
BSA	500 mg	10 mg/mL
Triton X-100	150 μ L	0.3%
Total	50 mL	

Store at 4°C for up to six months.

STEP-BY-STEP METHOD DETAILS

Transduction and selection of primary keratinocytes

⌚ Timing: 6–7 days

This step describes the engineering of primary human foreskin keratinocytes (HFK) to express GFP and HPV18 E7. In this example the cells are simultaneously transduced with two vectors: one encoding HPV18 E7 or an empty vector control and the second encoding GFP. Using two vectors alleviates the need to clone the gene or sgRNA of interest into a vector containing a fluorescent marker. It should be possible to engineer the cells using any approach that delivers the modification of interest plus a fluorescent marker gene.

1. Generate GFP and cell modification retroviruses and/or lentiviruses.
 - a. Identify retroviral or lentiviral vectors of interest.
 - i. Experiment requires GFP-expressing lentivirus or retrovirus, e.g., PHAGE-P-CMVt N-HA GFP.
 - ii. Experiment requires positive control lentivirus or retrovirus, such as HPV18 E7 expressing vector, e.g., Addgene #163311.
 - iii. Other lentiviruses/test conditions of interest should be determined by individual investigators.
 - b. Transfect suitable 293T cells, e.g., Lenti-X 293T (recommended passage: 10 or lower).
 - i. For each transfection, seed 2.5×10^6 293T cells into one 6 cm tissue culture dish. Use 293T transfection medium (lacks pen/strep).
 - ii. Let cells settle overnight.
 - iii. For each transfection, add 12 μ L of Trans-IT 293–400 μ L of Opti-MEM. Pipet to mix.
 - iv. Incubate at room temperature for 10 min.

- v. For each lentiviral transfection, add 2 μg of lentiviral vector, 1 μg of psPAX2, and 1 μg of pMD2.G. Pipet to mix.
- vi. Incubate at room temperature for 20 min.
- vii. For each retroviral transduction, add 2 μg of retroviral vector, 1 μg of pUMVC, and 1 μg of pCMV-VSV-G. Pipet to mix.
- viii. Incubate at room temperature for 20 min.
- ix. Add one transfection mix to one 6 cm plate of 293T cells, dropwise. Swirl to mix.
- x. Incubate for 24 h at 37°, 5% CO₂.
- c. Refeed the 6 cm plates.
 - i. Aspirate and disinfect the supernatant on each 6 cm plate.
 - ii. Replace with 2 mL of Monolayer Keratinocyte Serum Free Medium.
 - iii. Incubate for 24 h at 37°, 5% CO₂.
- d. Harvest lentivirus.
 - i. Collect supernatant containing lentiviral particles.
 - ii. Filter supernatant through 0.45 μm syringe filter to remove cell debris.
 - iii. Freeze aliquots of supernatant containing lentiviral particles at -80° for up to one year.

Note: A modified protocol describing retrovirus production can be found in.⁹ Detailed protocols for retroviral and lentiviral transduction are available on the Addgene website.

2. Prepare primary HFK cells. A detailed protocol for the isolation and cryopreservation of HFK is in.⁶
 - a. Thaw low passage primary HFK (recommended passage: 3 or lower).
 - i. Primary HFK should be used as soon as possible after initial isolation. We have had the best success with HFKs that were cryopreserved at passage two post-isolation or earlier.
 - b. The next time the cells are trypsinized and passaged, seed primary HFK for transduction or to maintain passage-matched cells for organotypic culture.
 - i. Using a 6 well tissue culture plate, seed 5×10^4 cells per well in 2 mL of monolayer keratinocyte serum free medium. Seed one well per cell condition of interest.
 - ii. Include an extra well to use as selection control for each drug-selectable marker being used. In this example the HPV18 E7 and control retroviral vectors confer G418 resistance and the GFP lentiviral vector confers puromycin resistance.
 - iii. Seed one 15 cm dish of HFK for every two planned organotypic cultures to use as the passage-matched, parental cells in later steps. Seed 1.5×10^5 HFK per plate.

Note: You will need 5×10^5 unmodified cells for each 0.5 cm² organotypic culture and 1×10^6 for each 1 cm² organotypic culture.

3. Transduce primary HFK cells.
 - a. 24 h after seeding, aspirate the existing media from wells of 6 well plates.
 - b. Add 1 mL of fresh monolayer keratinocyte serum free media containing 16 $\mu\text{g}/\text{mL}$ polybrene to each well of keratinocytes.
 - c. Add 750 μL of control or HPV18 E7 retrovirus and 250 μL of GFP lentivirus to appropriate wells.

Note: When using other retrovirus/lentivirus stocks, volume to add during this step must be determined empirically.

- d. Add 1 mL of monolayer keratinocyte serum free media to control wells.
- e. Swirl gently to mix.
- f. Incubate at 37°C with 5% CO₂ for 2 h.
- g. Replace media with fresh monolayer keratinocyte serum free media.
- h. Incubate at 37°C with 5% CO₂.

Table 1. Recommended antibiotic concentrations for selection of HFK

Antibiotic	Concentration in media ($\mu\text{g}/\text{mL}$)
Puromycin	0.5
G418	150
Blasticidin	3

4. Expand and select HFK cells.
 - a. 24 h post-transduction, passage transduced keratinocytes and seed all of the cells from a single transduced well into one 15 cm dish. One well will yield approximately 1.5×10^5 cells.
 - i. Culture the doubly transduced HFK in selection media with puromycin and G418.
 - ii. Culture one well of the control HFK in selection media containing puro and one well of the control HFK in G418.

Note: Typical antibiotic concentrations suitable for selection of primary HFK can be found in [Table 1](#), but the efficiency and timing of cell selection should be confirmed by individual investigators using their cells and antibiotic stocks.

- b. Refeed HFK with 18 mL of selection media every 48 h. Monitor selection progress by tracking the death of cells in the control plates.
 - i. 4–5 days post-selection (for G418), there should be no surviving cells in the control (untransduced) populations, indicating complete selection.
- c. Monitor GFP transduction by fluorescence microscopy. All of the doubly transduced cells should be GFP positive once the selection is complete ([Figure 1](#)).

Seeding dermis with fibroblasts

⌚ Timing: 3–4 days

This step describes how to seed devitalized dermis with primary human foreskin fibroblasts (HFF) before initiating organotypic epithelial cultures. To optimize the timing of organotypic culture setup, seed the HFF on the devitalized dermis the day after placing the HFK in selection. HFF in dermis and selected HFK should then be ready at approximately the same time.

5. Thaw sufficient dermis for experiment by placing frozen aliquot in conical tube in 37°C water bath.

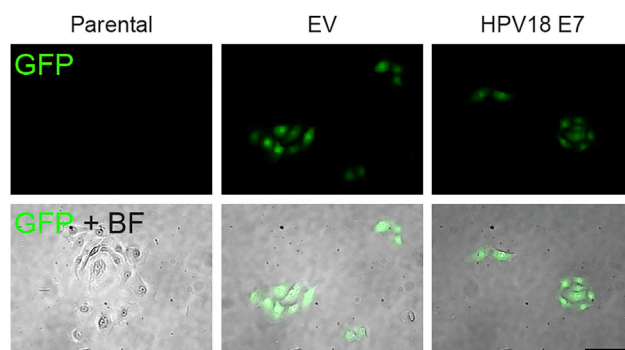


Figure 1. Assessment of successful transduction and selection

Primary HFK were transduced with a lentiviral vector encoding GFP and with a retroviral vector encoding either HPV18 E7 or the empty vector control (EV). GFP expression in engineered cell populations was assessed by fluorescence microscopy. Scale bar = $100 \mu\text{m}$.

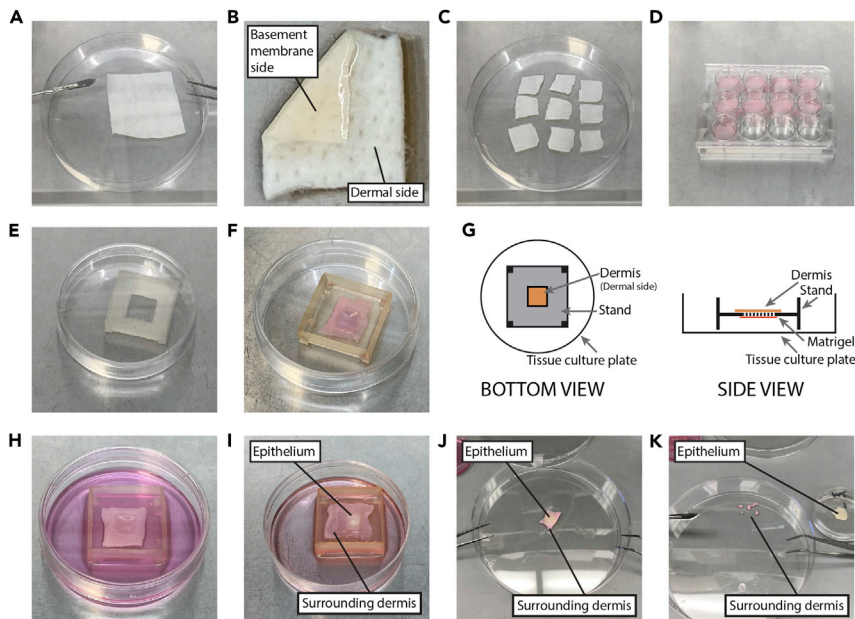


Figure 2. Establishing organotypic epithelial cultures

- (A) Thawed sheet of devitalized dermis.
 (B) Identification of basement membrane side and dermal side of devitalized dermis.
 (C) Devitalized dermis cut to individual organotypic culture sizes.
 (D) Devitalized dermis in 12-well plate (dermal side up) with HFF.
 (E) 3D printed organotypic culture stand.
 (F) Dermis stretched out over hole of organotypic culture stand.
 (G) Schematic of dermis and matrigel on stand, viewed from the bottom (left) and the side (right). Dashed line in side view indicates area of hole in stand.
 (H) Organotypic culture at the air-liquid interface.
 (I) Depiction of epithelial growth on an organotypic culture. Labels indicate the epithelium and the surrounding dermis.
 (J) Organotypic culture prior to trimming excess dermis.
 (K) Organotypic culture pre-fixing in formalin following trimming.

6. Pour both dermis and buffer into a 15 cm tissue culture plate.
7. Use autoclaved forceps to spread the dermis into a flat, even layer on the plate (Figure 2A).
8. Aspirate excess buffer.
9. Determine which side of the dermis is the basement membrane and which is dermal (Figure 2B).
 - a. The basement membrane side should look rough and slightly more tan in color.
 - b. The dermal side should look shiny and more white in color.

Note: Keep this orientation in mind, as it can be difficult to determine the orientation after media is added or on smaller pieces of dermis.

10. Cut the dermis to size (Figure 2C).
 - a. Using the forceps to guide the scalpel, cut the dermis into appropriately sized pieces. Each dermis piece should be a square, each edge of which is ~ 5 mm longer than the edge of the hole in the stand.

Note: It is helpful to have a sterile raft culture stand on hand to help you judge how big to cut the pieces of dermis.

Note: Video depictions of these and all subsequent dermis handling steps are viewable in Li et al.⁵

- b. Place the pieces of dermis into individual wells of a 12-well tissue culture plate with the dermal side facing up (Figure 2D).

Note: Unused pieces of dermis can be refrozen in PBS + penicillin and streptomycin at -80°C .

- c. Add 500 μL of HFF medium to each well.
11. Trypsinize and seed HFF (recommended passage: 6 or lower).
 - a. Trypsinize primary HFF by adding 2 mL of 0.05% Trypsin-EDTA solution per 10 cm plate of cells.
 - b. Inactivate trypsin by adding equal volume of HFF medium. Transfer cells to conical tube.
 - c. Centrifuge cells for 4 min at 290 RCF.
 - d. Resuspend cells in 2 mL fibroblast medium.
 - e. Seed 1×10^5 fibroblasts in 500 μL of medium on each piece of dermis. Final volume is 1 mL per well.
 - f. Culture the fibroblasts at 37°C on dermis.

Note: Fibroblasts and dermis can be co-cultured for 3–4 days, as needed to match HFK selection.

- g. Replace the media after 48 h.

△ CRITICAL: Be careful not to aspirate the dermis.

12. Check HFF density.
 - a. Look at the area in the well that is not covered by the dermis. The fibroblasts in these areas will be nearly 100% confluent when the dermis is ready for organotypic culture setup.

Establishing and maintaining organotypic epithelial cultures

⌚ **Timing:** 2 weeks

This step describes how to set up and maintain organotypic epithelial cultures for the purpose of monitoring cell fate decisions within a labeled subset of cells.

13. Prepare for organotypic culture setup (day before culture setup).
 - a. Autoclave a scalpel, at least two pairs of forceps, and the number of raft culture stands required for the experiment.
 - b. Thaw 200 μL of Matrigel per organotypic culture (see Note below) in an ice bucket at 4°C overnight.

Note: Although you will only need ~ 100 μL Matrigel per culture, thaw enough for 200 μL per culture.

14. Place dermis on stands.
 - a. For each raft, place one stand in a 6 cm dish (Figure 2E).
 - b. Using two pairs of forceps, stretch one fibroblast covered piece of dermis over the hole of each stand (Figures 2F and 2G Side View).
 - i. The dermis should be basement membrane side up / dermal side down on the stand.
 - ii. Make sure that there are no gaps around the edges of the hole.
 - iii. Aspirate any remaining liquid from the dermis prior to the Matrigel addition.
15. Plug the bottom of the stand with Matrigel.
 - a. Place the lid on all of the 6 cm dishes.

- b. Flip each covered dish so that the dermal side of the dermis is visible through the hole in the stand (Figure 2G Bottom View).

Note: Dermis will adhere to the stand with no intervention.

- c. Using pre-chilled tips, pipette about 100 μ L of Matrigel onto the bottom of each raft.
 - i. Ensure that the hole gets completely covered by Matrigel. Try not to produce any bubbles in the Matrigel.

Note: Because the Matrigel solidifies quickly, it can be helpful to pipette enough Matrigel for several rafts at once using a p1000.

- d. Allow Matrigel to solidify while preparing HFK.
16. Seed keratinocytes at the air-liquid interface.
 - a. Trypsinize engineered and parental HFK.
 - b. Inactivate trypsin by adding equal volume of E medium. Transfer cells to conical tube.
 - c. Centrifuge cells for 4 min at 290 RCF.
 - d. Resuspend cells in 2 mL E medium.
 - e. Prepare one 15 mL tube of cells for each raft culture to be established.
 - i. For each organotypic culture on an 0.5 cm² organotypic culture stand, aliquot 5×10^5 parental HFK into a fresh 15 mL conical tube.

Note: If using 1 cm² organotypic culture stand, use 1×10^6 parental HFK per culture.

- ii. Add the appropriate GFP-labeled HFK to each tube at the correct ratio. In this example, control- and HPV18 E7-expressing HFK were used at a ratio of 1:50 (modified to parental), so 1×10^4 modified cells were added to each tube of parental HFK.
- iii. Mix cells gently but thoroughly.
- iv. Centrifuge cells for 4 min at 290 RCF.
- v. Aspirate the media from the keratinocyte pellets, removing as much of the media as possible without disturbing the pellets.
- f. When the Matrigel is set, flip the 6 cm dishes containing the organotypic culture stands right side up.

Note: Tap the lids of the dishes to get the stands to drop if necessary.

- g. Working one culture at a time, resuspend the keratinocyte pellets in 40 μ L of E media.
- h. Seed all of the suspension in the center of the basement membrane side of the dermis on the organotypic culture stand.

Note: Be careful not to let the cell suspension spill off of the dermis. Using a lower volume for the resuspension can help to prevent cell loss at the time of culture setup.

17. Fill the plate surrounding the stand with E media until it just touches the bottom of the stand (around 3 mL) (Figure 2H).
18. Replace the media every 2–3 days.
19. Monitor the cultures for growth.

Note: A tan mass of cells will become visible in the center of the dermis in the first few days of culturing. The cells should expand to nearly fill the section of the dermis that is in contact with the liquid below (Figure 2I).



Table 2. Deparaffinization washes

Wash	Time
Xylene	3 min
Xylene	3 min
Xylene	3 min
100% Ethanol	3 min
100% Ethanol	3 min
95% Ethanol	3 min
70% Ethanol	3 min

Fixation of organotypic epithelial cultures

⌚ Timing: 1 day

This step describes how to fix organotypic epithelial culture tissues using 10% neutral buffered formalin.

20. Trim organotypic cultures.
 - a. Using a clean 15cm plate as a cutting surface, trim off the portions of the dermis that contain no epithelium.

Note: The epithelium is a thin tan/yellow layer on the basement membrane side of the dermis (Figure 2J).

21. **Optional:** Pre-fix the organotypic culture.
 - a. Add a thin layer of neutral buffered formalin (approximately 1 mL) into the bottom of a clean 6 cm dish.
 - b. Place the trimmed organotypic culture in this dish.
 - i. Tilt and submerge the organotypic culture into the formalin with forceps.
 - ii. Then ensure the culture is lying flat.
 - c. Leave the dish undisturbed at room temperature for 10 min (Figure 2K).
 - d. The pre-fixation step ensures that the organotypic culture does not curl during the prolonged fixation overnight.
22. Fix the organotypic raft cultures.
 - a. Place the trimmed organotypic culture in excess neutral buffer formalin—one jar per culture.
 - b. If the optional pre-fixation step was used, transfer the culture to the larger jar now.
 - c. Place in a dark, room temperature place for 24 h.
23. Dehydrate tissues in 70% ethanol, embed in paraffin, and cut sections onto slides.

Staining and imaging

⌚ Timing: 4 h

This step describes how to stain sections of the organotypic epithelial cultures by immunofluorescence.

24. Deparaffinize tissue sections.
 - a. Place slides at 55°C until the paraffin wax becomes clear (around 30 min–1 h).
 - b. Remove the paraffin from the slides by incubating the slides in the wash series described in Table 2.
 - c. Wash slides 5 times with water.

⏸ **Pause point:** The deparaffinized slides can be stored in water at 4°C for several days.

Table 3. Example primary antibody dilution

Component	Volume
Goat Serum	250 μ L
anti-IVL (Mouse monoclonal)	25 μ L (1:100)
anti-GFP (Rabbit polyclonal)	1.25 μ L (1:2000)
PBS	2223.75 μ L
Total	2.5 mL

25. Heat-induced epitope retrieval.
 - a. Fill a polypropylene coplin jar with Sodium Citrate antigen retrieval buffer and place it in the bowl of a pressure cooker.
 - b. Add 1–2 cm of water to the bowl around the jar.
 - c. Preheat the pressure cooker until the water inside comes to a boil.
 - d. Stop the pressure cooker, release the pressure, and place the slides in the coplin jar containing the antigen retrieval buffer.
 - e. Restart the pressure cooker and allow to incubate for 10 min after coming up to pressure.
 - f. Release the pressure. Remove the jar from the pressure cooker place into a wet ice bath.
 - g. Allow jar and slides to cool for 15 min.
26. Block.
 - a. Remove slides one at a time from the cooled coplin jar.
 - b. Tap the side of the slide gently on a paper towel to remove any residual liquid.
 - c. Using a PAP pen, draw a border around the tissue sections.
 - d. Wash the tissues with 100–200 μ L of 0.05% TWEEN 20 in PBS.
 - e. Aspirate the wash buffer and replace with histology blocking buffer spiked with 10% goat serum.
 - f. Incubate for 30–60 min.
27. Incubate in primary antibody.
 - a. Aspirate the blocking mixture.
 - b. Dilute primary antibodies in blocking buffer spiked with 10% goat serum (Table 3). Adjust the total volume of primary antibody dilution based on the number of tissues being stained.
 - c. Add 100–200 μ L of primary antibody dilution to the tissue sections.
 - d. Incubate at 4°C overnight.
28. Incubate in secondary antibody.
 - a. Aspirate the primary antibody mixture.
 - b. Wash the tissue sections with 0.05% TWEEN 20 in PBS three times for 5 min each.
 - c. Dilute secondary antibodies to blocking buffer spiked with 10% goat serum (Table 4). Adjust the total volume of secondary antibody dilution based on the number of tissues being stained.
 - d. Add 100–200 μ L of secondary antibody dilution to the tissue sections.
 - e. Incubate at room temperature for 1–2 h.
29. Mount coverslips.
 - a. Wash the tissue sections with 0.05% TWEEN 20 in PBS three times for 5 min each.
 - b. Add a drop of mounting media to the tissue section.
 - c. Slowly lower a cover slip onto the slide, taking care not to trap any bubbles.

Table 4. Example secondary antibody dilution

Component	Volume
Goat Serum	250 μ L
anti-Mouse IgG Alexa Fluor 488 (Goat polyclonal)	10 μ L (1:250)
anti-Rabbit IgG Alexa Fluor 594 (Goat polyclonal)	10 μ L (1:250)
Hoechst (diluted 1:10 in PBS)	2.5 μ L (1:10,000 final)
PBS	2223.75 μ L
Total	2.5 mL

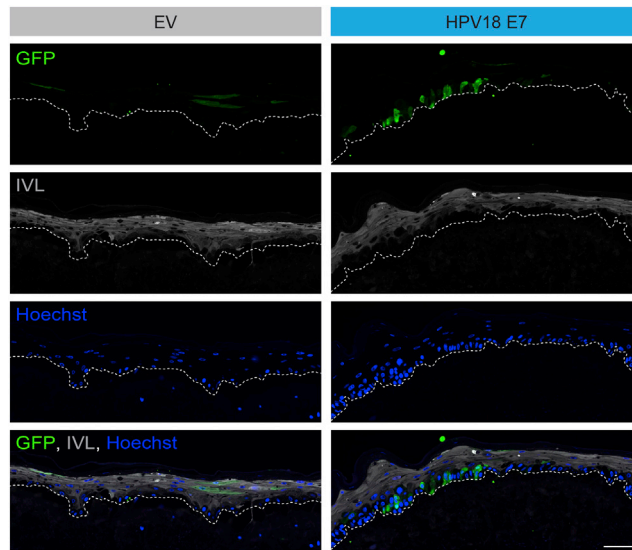


Figure 3. Example images of organotypic epithelial cultures imaged for GFP+ cell fate

Organotypic epithelial cultures were grown from GFP-labeled cells mixed with unmodified parental HFK at a dilution of 1:50 (GFP-labeled:parental). GFP-expressing cells of one epithelial culture also express HPV18 E7 from a retroviral vector and GFP-expressing cells from the other express the empty control vector (EV). Note that the majority of GFP+ cells in the EV condition are found in the suprabasal layers (IVL+), while most of the GFP+ cells in the HPV18 E7-expressing condition are found in the basal layer (IVL-). Scale bar = 100 μ m.

- d. Seal the coverslip to the slide with nail polish.
30. Image.
- a. Image the organotypic culture tissue section using a fluorescent microscope (Figure 3).

Note: To record enough GFP+ cells for quantification, we recommend taking many images (or one stitched image) that cover the entire length of the organotypic culture section.

EXPECTED OUTCOMES

Control keratinocytes are expected to be randomly distributed through the layers of the epithelium. There are many more cells in the suprabasal layers than in the basal layer, so it is expected that control (vector transduced) cells will be found predominantly in the suprabasal layers (Figure 3). In our cultures, 70%–90% of control cells are typically in the suprabasal layers and 10%–30% remain in close proximity to the basement membrane. Certain genetic modifications alter this ratio. For example, we have observed that YAP1 activation downstream of HPV18 E7 is associated with the presence of about 80% of labeled cells in the basal cell layer¹ (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

This section describes how to quantify images by visually classifying each GFP+ cell as a basal or a suprabasal cell. In this example, our focus was on whether cells were in the basal layer. GFP+ cells were classified as ‘basal’ (IVL- and adjacent to the basement membrane) or ‘suprabasal’ (IVL+ and far from the basement membrane). There are many methods used to manually quantify images. In this section, we describe a method that uses FIJI macros to help to minimize bias, improve record keeping, and reduce the time it takes to count a large number of images. Following visual classification, the tally of GFP+ cells belonging to each compartment can be expressed as a percentage of the total number of GFP+ cells per organotypic culture. Statistical analysis can be performed using a t-test for a two-condition experiment or an ANOVA with multiple t-tests for a multi-condition experiment.

1. Organize for quantification.
 - a. Convert all images to be used for quantification to composite images and place these images in a single folder.
2. Load the "Mark Counting Quantification" toolset in FIJI.
 - a. Click ">>" in the FIJI toolbar.
 - b. Select "Mark Counting Quantification."
3. Randomize the names of the images for quantification.
 - a. In FIJI, click Plugins > Macros > Randomize Folder of Images.
 - b. When prompted, select the folder of images to be quantified as the input folder. Then, select a new folder as the location to output the renamed images.

```
CountDots()

macro "Randomize Folder of Images" {
  setBatchMode(true);
  Dialog.create("Choose Input");
  Dialog.addMessage("Choose Directory For Input");
  Dialog.show();
  dir1 = getDirectory("Choose Directory For Input");
  Dialog.create("Choose Output");
  Dialog.addMessage("Choose Directory For Output");
  Dialog.show();
  Odir = getDirectory("Choose Directory For Output");
  sep=File.separator;
  File.makeDirectory(Odir+"Images"+sep);
  list1 = getFileList(dir1);
  for (i = 0; i < list1.length; i++) {
    Rando(dir1, Odir, list1[i]);
  }
  selectWindow("Log");
  saveAs("Text", Odir+sep+"Image Decoder.csv");
  run("Close");
  setBatchMode(false);
}

function Rando(dir1, Odir, name1) {
  RandomCode = "Image ";
  for (r = 0; r < 15; r++) {
    RandomCode = RandomCode + d2s(random*9, 0);
  }
  path=dir1+name1;
  open(path);
  path=Odir+sep+"Images"+sep+RandomCode+".tif";
}
```

```
    saveAs("Tiff", path);

    close();

    splname=split(name1, ".");

    print(splname[0]+", ", RandomCode);
}

macro "Save Overlays" {
    setBatchMode(true);

    Dialog.create("Choose Input");

    Dialog.addMessage("Choose Directory For Input");

    Dialog.show();

    dir1 = getDirectory("Choose Directory For Input");

    Dialog.create("Choose Output");

    Dialog.addMessage("Choose Directory For Output");

    Dialog.show();

    Odir = getDirectory("Choose Directory For Output");

    sep=File.separator;

    list1 = getFileList(dir1);

    for (i = 0; i < list1.length; i++) {
        SaveOverlay(dir1, Odir, list1[i]);
    }

    setBatchMode(false);
}

function SaveOverlay(dir1, Odir, name1) {

    path=dir1+name1;

    open(path);

    Stack.setActiveChannels("011");

    Stack.setActiveChannels("001");

    Stack.setActiveChannels("000");

    splname=split(name1, ".");

    path=Odir+sep+splname[0]+".jpg";

    saveAs("Jpeg", path);

    close();
}

macro "Sort and Count Marks" {

    setBatchMode(true);

    dir1 = getDirectory("Choose Directory For Input");

    Odir = getDirectory("Choose Directory For Results Output");

    list1 = getFileList(dir1);
```

```
//Get Number of colors from user
nAry = newArray("1", "2", "3", "4", "5", "6")
Dialog.create("Choose Number of Colors");
Dialog.addChoice("Number of Colors:", nAry);
Dialog.show();
nColor = parseInt(Dialog.getChoice());
//Identify colors in use
cNameAry = newArray("Red", "Green", "Blue", "Cyan", "Magenta", "Yellow");
userColorArray = newArray(nColor);
OutputDirArray = newArray(nColor);
sep=File.separator;
for (i = 0; i < nColor; i++) {
    Dialog.create("Choose Color "+i);
    Dialog.addChoice("Color "+i+":", cNameAry);
    Dialog.show();
    userColorArray[i] = Dialog.getChoice();
    OutputDirArray[i] = ""+Odir+userColorArray[i]+sep+"";
    File.makeDirectory(OutputDirArray[i]);
}
Dialog.create("Set Min. Dot Size");
Dialog.addNumber("Min. Size (pixel^2):", 0);
Dialog.show();
fMin = Dialog.getNumber();
run("Color Threshold...");
wait(1000);
for (j = 0; j < nColor; j++) {
    //Sort dots in each image for per color
    for (i = 0; i < list1.length; i++) {
        Sort(dir1, OutputDirArray[j], list1[i], userColorArray[j]);
    }
    //count dots in each image of each color
    list2 = getFileList(OutputDirArray[j]);
    for (i = 0; i < list1.length; i++) {
        Measure(OutputDirArray[j], list2[i], fMin);
    }
    IJ.renameResults("Summary", "Results");
    path=Odir+userColorArray[j]+" Counts.csv";
    saveAs("Results", path);
}
```

```
selectWindow("Results");
run("Close");
}
selectWindow("Threshold Color");
run("Close");
setBatchMode(false);
}
function Sort(dir1, Odir, name1, Color1) {
    path=dir1+name1;
    open(path);
    run("Color Threshold...");
    min=newArray(3);
    max=newArray(3);
    filter=newArray(3);
    a=getTitle();
    run("HSB Stack");
    run("Convert Stack to Images");
    selectWindow("Hue");
    rename("0");
    selectWindow("Saturation");
    rename("1");
    selectWindow("Brightness");
    rename("2");
    //Set Hue parameters
    if (Color1 == "Green"){
        min[0]=74;
        max[0]=94;
    }
    if (Color1 == "Blue"){
        min[0]=164;
        max[0]=186;
    }
    if (Color1 == "Cyan"){
        min[0]=119;
        max[0]=139;
    }
    if (Color1 == "Magenta"){
        min[0]=205;
```

```

    max[0]=240;
  }
  if (Color1 == "Yellow"){
    min[0]=28;
    max[0]=48;
  }
  filter[0]="pass";
  if (Color1 == "Red"){
    min[0]=10;
    max[0]=245;
    filter[0]="stop";
  }
  //Set saturation parameters
  min[1]=0;
  max[1]=255;
  filter[1]="pass";
  //Set brightness parameters
  min[2]=124;
  max[2]=255;
  filter[2]="pass";
  //Run threshold
  for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
  }
  //Create new image
  imageCalculator("AND create", "0", "1");
  imageCalculator("AND create", "Result of 0", "2");
  for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
  }
  selectWindow("Result of 0");
  close();
  selectWindow("Result of Result of 0");
  rename(a);

```

```
//Color Thresholding

run("8-bit");

setAutoThreshold("Default");

setOption("BlackBackground", false);

run("Convert to Mask");

splname=split(name1, ".");

suf=".tif";

path=Odir+splname[0]+" "+Color1+suf;

saveAs("Tiff", path);

close();

}

function Measure(dir1, name1, fMin) {

    path=dir1+name1;

    open(path);

    run("Set Measurements...", "area redirect=None decimal=3");

    script="size="+fMin+" show=Nothing summarize";

    run("Analyze Particles...", script);

    close();

}
```

- c. Then, select a new folder as the location to output the renamed images.
- d. This will generate folder of images with randomized names and a spreadsheet that can be used to decode the results.
4. Mark each GFP+ cell as basal or suprabasal.
 - a. In FIJI, open the brush options tool and the channels tool (Figure 4A).
 - i. Check the box next to "Paint on overlay" in the brush options tool.
 - ii. The channels tool can be used to turn on and off individual channels of composite images during analysis to better assess classification for each cell.
 - b. Open images with randomized names one at a time (Figure 4B).
 - i. Using the brush tool, mark each GFP positive cell with a single dot of a color that represents the layer in which it resides. In this example, basal cells were marked with yellow dots and suprabasal cells were marked with magenta dots (Figure 4C).
 - ii. Save and close the image.
 - iii. Repeat for each image included in the analysis.
5. Save the overlays containing the marks as separate images (Figure 4D).
 - a. In FIJI, click Plugins > Macros > Save Overlays.
 - b. When prompted, select the folder of marked images as the input folder.
 - c. Then, select a new folder as the location to output the overlay images.
6. Count the marks of each color (Figure 4E).
 - a. In FIJI, click Plugins > Macros > Sort and Count Marks.
 - b. When prompted, select the folder of overlay images as the input folder.
 - c. Then, select a new folder as the location to output the results.
7. Decode the results (Figure 4F).
8. Pool the counts from multiple images per organotypic culture to calculate the percentage of GFP+ cells in the basal layer (Table 5) (Figure 4G).

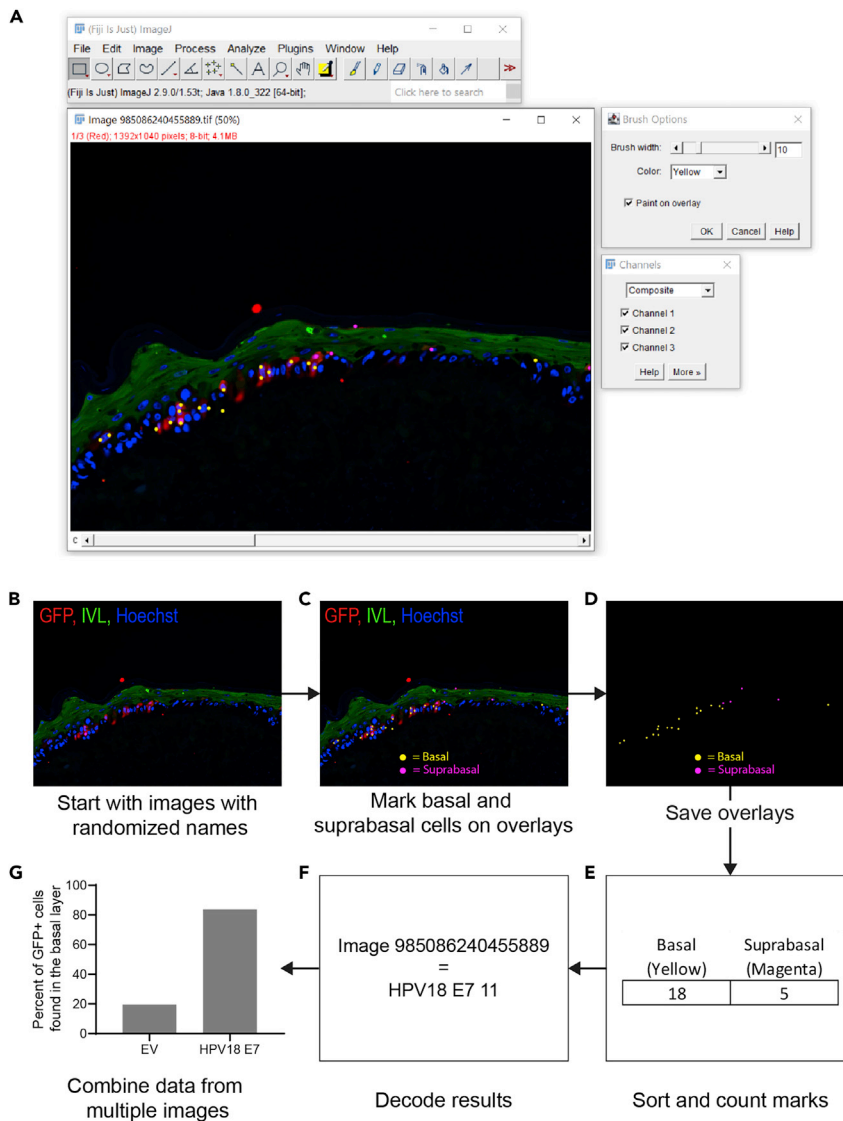


Figure 4. Manually quantifying the percentage of GFP+ cells found in each layer

(A) Fiji tools required for quantification.

(B) Three channel image of organotypic culture prior to quantification. GFP is represented in red, IVL is represented in green, and Hoechst is represented in blue.

(C) Three channel image of organotypic culture after being manually marked for quantification. GFP is represented in red, IVL is represented in green, and Hoechst is represented in blue. Yellow marks represent GFP+ cells determined to be in the basal layer, and magenta marks represent cells determined to be in the suprabasal layers.

(D) Overlay containing marks for quantification.

(E) Representative basal and suprabasal GFP+ cell counts from an individual image.

(F) Representative decoding of an image identity.

(G) Quantification of the percentage of GFP+ cells found in the basal layer of one EV organotypic culture and one HPV18 E7 organotypic culture. These data were derived from 12 or 13 separate images of the EV and HPV18 E7 organotypic cultures respectively.

LIMITATIONS

The behavior of HFK from different donors may vary in this assay. We recommend performing these experiments with HFK from at least two different donors. There can be variation in epithelial stratification and differentiation between experiments, resulting in suprabasal layers of varying sizes. The thickness of the stratum corneum can be especially variable.

Table 5. Representative quantification data

Image name	Image code	Basal (yellow)	Suprabasal (magenta)
EV 01	Image 009283320423764	0	2
EV 02	Image 379941907124882	4	2
EV 03	Image 177913711528655	1	6
EV 04	Image 546868629435524	0	6
EV 05	Image 727739758333724	0	3
EV 06	Image 678212414386838	0	0
EV 07	Image 913130974826601	1	1
EV 08	Image 341413337374187	0	2
EV 09	Image 546367357367461	1	1
EV 10	Image 439374260514271	1	5
EV 11	Image 033049662745253	0	3
EV 12	Image 683793671448871	0	2
HPV18 E7 01	Image 225108124041607	23	1
HPV18 E7 02	Image 713767677406711	10	2
HPV18 E7 03	Image 065238709164222	14	0
HPV18 E7 04	Image 651168633158131	17	3
HPV18 E7 05	Image 262629175740838	9	11
HPV18 E7 06	Image 370116172471826	7	1
HPV18 E7 07	Image 565739032218556	15	2
HPV18 E7 08	Image 823968877536641	29	6
HPV18 E7 09	Image 483388753643222	13	0
HPV18 E7 10	Image 754919199781356	43	5
HPV18 E7 11	Image 985086240455889	18	5
HPV18 E7 12	Image 402959625423145	5	2
HPV18 E7 13	Image 672284281852970	18	5
EV (Total)	–	8	33
HPV18 E7 (Total)	–	221	43
EV (%)	–	19.51%	80.49%
HPV18 E7 (%)	–	83.71%	16.29%

TROUBLESHOOTING

Problem 1

Poor overall organotypic culture growth (step 19).

Potential solution

This may be due to low proliferative potential of parental, unmodified keratinocytes at the time of seeding. Be sure to start the process at very low passage. Minimize contact with high calcium media or media containing serum. Be sure that the keratinocytes are never overconfluent prior to seeding the organotypic cultures.

Problem 2

Difficulty finding GFP+ cells when imaging (step 30).

Potential solution

This could result from a ratio of engineered:parental cells that is too low. Be sure to optimize the ratio of engineered to parental keratinocytes by testing several dilutions for a given condition. We have used ratios ranging from 1:200 (engineered:parental) to 1:25 depending on the proliferative capacity of the engineered cells.

This could also be caused by low level staining of GFP expressed from the labeling vector in an experiment. When testing a new GFP vector, include a culture with no GFP+ cells and a culture with only GFP+ cells as controls. Sections of these control cultures can be used to optimize staining

for GFP expression. While we have had success with delivering GFP in a variety of retroviral and lentiviral vectors that result in different expression levels, expect a lower signal-to-noise ratio from vectors that express lower levels of GFP.

RESOURCE AVAILABILITY

Lead contact

Requests for further information or for resources and reagents should be directed to and will be fulfilled by the lead contact, Elizabeth White (eawwhite@penncellpress.upenn.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

This study did not generate or analyze datasets. Code for Fiji plugin is supplied in text format above and in [Data S3](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2023.102101>.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, and design, J.H., C.A.N., T.W.R., E.A.W.; acquisition of data, J.H.; analysis and interpretation of data, J.H., E.A.W.; drafting or revising the article, J.H., C.A.N., T.W.R., E.A.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Hatterschide, J., Castagnino, P., Kim, H.W., Sperry, S.M., Montone, K.T., Basu, D., and White, E.A. (2022). YAP1 activation by human papillomavirus E7 promotes basal cell identity in squamous epithelia. *Elife* 11, e75466. <https://doi.org/10.7554/eLife.75466>.
- Ridky, T.W., Chow, J.M., Wong, D.J., and Khavari, P.A. (2010). Invasive three-dimensional organotypic neoplasia from multiple normal human epithelia. *Nat. Med.* 16, 1450–1455. <https://doi.org/10.1038/nm.2265>.
- Egolf, S., Aubert, Y., Doepner, M., Anderson, A., Maldonado-Lopez, A., Pacella, G., Lee, J., Ko, E.K., Zou, J., Lan, Y., et al. (2019). LSD1 inhibition promotes epithelial differentiation through derepression of fate-determining transcription factors. *Cell Rep.* 28, 1981–1992.e7. <https://doi.org/10.1016/j.celrep.2019.07.058>.
- Duperret, E.K., Dahal, A., and Ridky, T.W. (2015). Focal-adhesion-independent integrin- α regulation of FAK and c-Myc is necessary for 3D skin formation and tumor invasion. *J. Cell Sci.* 128, 3997–4013. <https://doi.org/10.1242/jcs.175539>.
- Li, J., and Sen, G.L. (2015). Generation of genetically modified organotypic skin cultures using devitalized human dermis. *J. Vis. Exp.* e53280. <https://doi.org/10.3791/53280>.
- Fehrmann, F., and Laimins, L.A. (2005). Human papillomavirus type 31 life cycle methods for studying using tissue culture models. *Methods Mol. Biol.* 292, 317–330.
- Galligan, J.T., Martinez-Noël, G., Arndt, V., Hayes, S., Chittenden, T.W., Harper, J.W., and Howley, P.M. (2015). Proteomic analysis and identification of cellular interactors of the giant ubiquitin ligase HERC2. *J. Proteome Res.* 14, 953–966. <https://doi.org/10.1021/pr501005v>.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. <https://doi.org/10.1038/nmeth.2019>.
- White, E.A., Sowa, M.E., Tan, M.J.A., Jeudy, S., Hayes, S.D., Santha, S., Münger, K., Harper, J.W., and Howley, P.M. (2012). Systematic identification of interactions between host cell proteins and E7 oncoproteins from diverse human papillomaviruses. *Proc. Natl. Acad. Sci. USA* 109, E260–E267. <https://doi.org/10.1073/pnas.1116776109>.