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Using Organotypic Epithelial Tissue Culture to Study the Human Papillomavirus Life Cycle

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Abstract

Human papillomaviruses (HPVs) are small double stranded DNA viruses that are associated with greater than 95% of cervical cancers and 20% of head and neck cancers. These cancers arise from persistent infections in which there is continued expression of the HPV E6 and E7 oncogenes, often as a consequence of integration of HPV DNA into the host genome. Such cancers represent “dead ends” for the virus as integration disrupts the viral genome and because the cancers are defective in normal epithelial differentiation, which is required for production of progeny papillomavirus. In order to study the full viral life cycle, from establishment to maintenance to the productive stage, our lab makes use of the organotypic epithelial tissue culture system. This system allows us to mimic the three-dimensional structure of epithelia whose differentiation is tightly linked to the completion of the HPV viral life cycle. In this chapter we describe how various aspects of the HPV life cycle are monitored in raft cultures making use of an immortalized keratinocyte cell line.

Keywords

papillomavirus; life cycle; organotypic epithelial tissue culture; keratinocyte

Introduction

In this unit we provide a description of the use of an immortalized human foreskin keratinocyte cell line in organotypic epithelial culture to study the life cycle of human papillomaviruses (HPVs). Included are protocols for creating the organotypic epithelial culture as well as monitoring different aspects of the HPV life cycle in raft cultures (i.e. unscheduled DNA synthesis, differentiation, viral amplification and late protein expression).

CAUTION: Human papillomavirus (HPV) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (APPENDIX 1B) for more information.

Basic Protocol 1: Generating the organotypic epithelial raft cultures

In this protocol, a detailed methodology for producing stratified squamous epithelia using an organotypic epithelial culturing approach is described. This epithelial culture is commonly referred to as a “raft” culture because one “floats” the keratinocyte cell culture at the air/liquid interface. By culturing HPV-harboring keratinocytes on raft cultures, one can recapitulate all stages of the viral life cycle. Raft culturing is extremely cumbersome and requires a great degree of adherence to details. It is also very important to use proper sterile technique and high-quality reagents.

Materials

Transwell inserts (Costar #3450)
 Deep well trays (Corning #355467)
 Rat tail collagen type 1 (Millipore #08-115)
 10X F12 media (ThermoFisher Scientific #21700-075)
 Human fibroblasts for embedding in dermal equivalent (EF-1-F from Lambert lab)
 Fibroblast media (see recipe)
 Keratinocytes harboring HPV genome of interest
 Keratinocyte plating media (see recipe)
 Cornification media 1 (see recipe)
 Cornification media 2 (if so needed; see recipe)
 Cotton pads cut into 1” squares and autoclaved (VWR #21427-393)
 1,2-dioctanoyl-*sn*-glycerol, [C8:O] (Cayman Chemicals #62225)
 5-bromo-2'-deoxyuridine, [BrdU] (Sigma #B5002)
 Bacto-agar
 10% buffered formalin
 Tissue-Tek cassettes (Sakura #4118-01)

Preparation of collagen raft (dermal equivalent)

1. Remove the transwell inserts from the packaging with sterile forceps, and place into the wells of the deep well trays.
2. Trypsinize human fibroblasts and resuspend in fibroblast media to a final concentration of 7.5×10^5 cells/ml. One confluent 10 cm dish of fibroblasts is enough for 12 rafts.
3. Prepare collagen pre-mix (25 ml is sufficient for 6 transwell inserts or rafts, scaling up or down as needed) by mixing the following reagents in 50 ml centrifuge tube. Avoid bubbles and keep tube on ice as collagen will

solidify at room temperature. Bubbles can be removed by centrifugation at 1500 RPM for 5 minutes at 4°C if necessary.

a. 10X F12 media	2.5 ml
b. 10N NaOH	6 µl
c. Pen/Strep	250 µl
d. Fetal Bovine Serum (FBS)	2.5 ml
e. Rat tail collagen	20 ml

4. Plate 1 ml of collagen pre-mix onto the bottom of each transwell insert, tilting to coat evenly. Allow to solidify at room temperature (approximately 5 minutes).
5. Add 600 µl of the 7.5×10^5 cells/ml (4.5×10^5 cells) of human fibroblasts to the remaining collagen mixture, mixing thoroughly and avoiding air bubbles as before.
6. Layer 2.5 ml of the fibroblast/collagen mixture over each of the 1 ml gels and allow to solidify in incubator for at least 30 minutes.
7. Once the rafts have solidified, add 20 ml fibroblast media to each deep well containing a transwell and incubate at 37°C, 5% CO₂.
8. Gels will contract to appropriate shape within 4–7 days during which time the media need not be changed.

Raft culturing—In our lab, keratinocytes are plated on day 0, fed once on day 2, lifted on day 4 and harvested on day 15. The following describes the 16-day process from plating to harvest.

On day 0 (plating keratinocytes)

- 9 Trypsinize keratinocytes to be plated onto rafts, pellet cells, and resuspend in keratinocyte plating media to a final concentration of 1.4×10^6 cells/ml.
- 10 Aspirate media from outer well and carefully remove the media from inner transwell by using a pipette. Use caution to avoid disturbing the dermal equivalent.
- 11 Layer 150 µl of keratinocyte suspension (2.1×10^5 cells) onto collagen raft.
- 12 Incubate for 2 hours at 37°C and 5% CO₂ to allow cells to attach to collagen raft.
- 13 Carefully add 20 ml keratinocyte plating media to each deep well. Be sure to add media to the outside of the transwell, not to the inner well as to avoid disturbing the recently plated keratinocytes.

On day 2 (feeding rafts)

- 14 Aspirate media from outer well, and carefully remove media from inner transwell with a pipette.
- 15 Feed rafts by adding 20 ml keratinocyte plating media to outer well.

On day 4 (lifting rafts)

- 16 Aspirate media from outer well, and carefully remove media from inner transwell with a pipette.
- 17 Using sterile forceps, lift transwell and place 4 cotton pads onto the risers of each well creating a platform upon which the insert will be placed to expose the raft to the air/liquid interface.
- 18 Place transwell onto cotton pads and feed with 11 ml cornification media 1 containing 10 μ M 1,2-dioctanoyl-*sn*-glycerol [C8:O]. Keratinocytes should be exposed to air for the remainder of the process and fed only via media absorption from the bottom through the cotton pads.

On day 6, 8, 10, 12, and 14 (feeding rafts)

- 19 Aspirate media from outer well, and carefully remove media from inner transwell with a pipette.
- 20 Add 10 ml cornification media 1 containing 10 μ M C8:O

On Day 15 (BrdU labeling)

- 21 Aspirate media from outer well.
- 22 Add 9–10 ml cornification media 1 containing 10 μ M C8:O and 10 μ M BrdU and allow to incubate for 8 hours at 37°C and 5% CO₂.

Raft harvesting—Our lab harvests rafts after 15 days. We have determined that this length of time is sufficient for completion of the full HPV viral life cycle. If the researcher wishes to extend the culturing beyond 15 days, switch to cornification media 2 supplemented with 10 μ M C8:O for feeding.

- 23 While rafts are being labeled with BrdU, prepare fixation solution (2% agar, 1% formalin) as follows:

2 g bacto-agar

90 ml ddH₂O

Boil above mixture **slowly** in microwave. Agar will flash and boil over if boiled rapidly.

- 24 Once agar has gone into solution, place into 65°C water bath to cool, but still remain molten. Using fixation solution that is too hot can damage the raft culture.
- 25 Add 10 ml 10% buffered formalin and mix prior to use.

- 26 After the 8 hour BrdU incubation, aspirate the medium from outer well of raft cultures.
- 27 Remove transwells from the deep-well plates. Cut the bottom transwell membranes from the plastic support by running along the outer perimeter with a scalpel; carefully remove upper plastic support of transwells (Figure 1A and 1B).
- 28 Plate a portion of fixation solution (3–4 mL/raft) onto a clean glass plate, creating a cushion like base slightly larger than the raft culture.
- 29 Carefully lift rafts off of transwell membrane and place them onto the fixation solution (Figure 1B,C).
- 30 Add fixation solution on top of the rafts to embed them (Figure 1D), doing your best to encase the raft in molten agar. Ensure that no portions of the raft are left uncovered with agar.
- 31 Wrap glass plates with foil or saran wrap to avoid drying.
- 32 Store at 4°C overnight.
- 33 Cut fixation solution-embedded rafts out with a scalpel and place into Tissue-Tek cassettes.
- 34 Soak rafts in the Tissue-Tek cassettes in 10% formalin and keep them at 4°C overnight.
- 35 Replace formalin with 70% ethanol. Store fixed rafts in 70% ethanol.
- 36 Rafts are ready for paraffin embedding and sectioning.

Basic Protocol 2: Analysis of Unscheduled DNA Synthesis by BrdU staining using Immunohistochemical Detection

In this section we describe the protocol for performing immunohistochemistry (IHC) on raft cultures that are formalin fixed and paraffin embedded. This protocol can be used for monitoring levels of expression of multiple cellular and viral antigens (e.g. BrdU, MCM7, keratin 10, involucrin, loricrin, as well as HPV E1E4 and L1). Immunofluorescence (IF) detection is described in the Alternate Protocol.

Materials

Coplin jar or staining dish
Xylenes
Graded alcohols (100%, 95%, 80% and 70%)
1X PBS
3% H₂O₂
Antigen retrieval solution (choose one):

10 mM citrate buffer pH 6.0
2X SSC
TE pH 9.0
Proteinase K
Pepsin
2 N HCl
Hydrophobic marker or PAP pen
Blocking serum (host species of 2° antibody)
Humidified chamber
α-BrdU antibody (Millipore #NA-61)
VECTASTAIN Elite ABC Kit, Universal (Vector Labs #PK-6200)
DAB detection kit (Vector Labs #SK-4100)
Vector Hematoxylin QS (Vector Labs #H-3404)
Coverslips
Cytoseal XYL

Optional step: Bake slides overnight at 65°C. Prior to tissue rehydration, slides can be baked overnight at 65°C in warming or hybridization oven. This may help tissue to remain adhered to slide during the harsh antigen retrieval step.

Note: Once slides have been rehydrated it is very important to avoid allowing the tissue to dry out. This can lead to high background staining.

1. Place slides into Coplin jar or staining dish. A Coplin jar will allow for the use of smaller volumes of reagents (40 ml) versus a staining dish (200 ml).
2. Rehydrate slides as follows:
 - 2 changes of xylenes for 5 minutes each
 - 2 changes of 100% ethanol for 3 minutes each
 - 95% ethanol for 3 minutes
 - 80% ethanol for 3 minutes
 - 70% ethanol for 3 minutes
 - ddH₂O for 3 minutes
 - 1X PBS for 5 minutes
3. Block endogenous peroxidase by incubating slides in 3% H₂O₂ for 20 minutes at room temperature.
4. Wash slides three times in 1X PBS for 5 minutes each wash.

5. For antigen retrieval, place slides into microwave safe staining dish and fill to top with 10 mM citrate buffer pH 6.0.
6. Boil for 20 minutes (3 minutes on high, 17 minutes on 70% power), adding fresh buffer every 5 minutes, making sure liquid level **never** goes below tissue.
7. After microwaving, leave slides in hot buffer on bench top and allow to cool for at least 45 minutes.
8. Wash slides three times in 1X PBS for 5 minutes each wash.
9. Incubate slides for 20 minutes in 2N HCl at room temperature.
10. Wash slides three times in 1X PBS for 5 minutes each wash.
11. Circle tissue section with PAP pen, or other hydrophobic marker.
12. For antigen blocking, apply 100 μ l of blocking solution (5% serum in 1X PBS) to circled tissue section and spread evenly. Serum is determined by the host species of the secondary antibody being used for detection (i.e. horse serum if the secondary antibody was made in horse).
13. Incubate slides for one hour in blocking solution at room temperature in a humidified chamber.
14. Aspirate blocking solution, do not wash.
15. To incubate primary antibody, dilute α -BrdU antibody (1:50) in the same blocking solution as above.
16. Apply 100 μ l of diluted primary antibody to tissue section, spreading evenly.
17. Incubate in a humidified chamber at 4°C overnight. Include a no primary antibody condition as a control for antibody specificity and background signal.
18. Wash slides three times in 1X PBS for 5 minutes each wash.
19. Dilute secondary antibody from VECTASTain kit (1:100) in same blocking solution as above.
20. Apply 100 μ l of diluted secondary antibody to circled tissue section, spreading evenly.
21. Incubate in humidified chamber for one hour at room temperature.
22. Wash slides three times in 1X PBS for 5 minutes each wash.
23. Dilute ABC reagent, following manufacturer's protocol.
24. Apply 100 μ l of diluted ABC reagent to tissue section, spreading evenly.
25. Incubate in humidified chamber for 30 minutes at room temperature.
26. Wash slides three times in 1X PBS for 5 minutes each wash.

27. Develop slides with DAB or other peroxidase detection kit following manufacturer's protocol.
28. Follow development under microscope.
BrdU staining should be intense within 2 minutes.
No primary control will provide level of background staining in experiment.
29. Wash slides three times in 1X PBS for 5 minutes each wash.
30. Counterstain with Hematoxylin QS following manufacturer's protocol.
31. Dehydrate slides as follows
 - ddH₂O for 3 minutes
 - 70% ethanol for 3 minutes
 - 80% ethanol for 3 minutes
 - 95% ethanol for 3 minutes
 - 2 changes of 100% ethanol for 3 minutes each
 - 2 changes of xylenes for 3 minutes each
32. Keep slides in xylenes while applying coverslip.
33. Working in groups of 4–5, place wet slide onto paper towel, and apply one drop of Cytoseal XYL to tissue.
34. Dip coverslip into xylene and lay onto tissue, allowing Cytoseal XYL to spread via xylenes. Apply slight pressure to slide to remove bubbles trapped between tissue and coverslip.
35. Allow slides to air dry.

Alternate Protocol: BrdU staining using Immunofluorescent Detection

Here we describe how to modify Basic Protocol 2 in order to use fluorescently labeled antibodies for detection.

Materials

α-mouse AlexaFluor conjugated secondary antibody (ThermoFisher)

Hoescht Dye 33342 (Sigma, #B2261)

Anti-fade mounting medium

1. Omit steps 3 and 4 above: No need to block endogenous peroxidase when detection is by fluorescence.
2. Secondary antibody incubation:

- a. Dilute α -mouse AlexaFluor (Life Technologies) antibody 1:1000 in blocking solution.
 - b. Apply 100 μ l of diluted secondary antibody to circled tissue section, spreading evenly.
 - c. Incubate in humidified chamber for one hour at room temperature.
3. Omit steps 23–35 replacing with below:
 - a. Counterstain with diluted Hoescht's Dye for 10 minutes at room temperature.
 - b. Wash slides three times in 1X PBS for 5 minutes each wash.
 - c. Apply anti-fade mounting media to tissue and add coverslip.
4. Store slides protected from light at -20°C until imaging.

Basic Protocol 3: Viral DNA Amplification by Fluorescence *in situ* Hybridization (FISH)

HPV DNA is amplified in the terminally differentiating compartment of stratified epithelia. This amplification is another hallmark of the productive phase of the viral life cycle and can be directly visualized in raft cultures of HPV-positive NIKS through the use of HPV-specific *in situ* hybridization. While our initial approach made use of radioactively labeled *in situ* hybridization probes (Flores et al., 1999) we have now turned to the use of fluorescence *in situ* hybridization (FISH) as described below and illustrated in Figure 2.

Materials

Xylenes
 Ethanol (70%, 80%, 95% and 100%)
 10 mM citrate buffer pH 6.0
 10X NT buffer
 10X nucleotide stock
 0.4 mM dTTP
 DNase I stock solution
 0.1 M β -mercaptoethanol
 E.Coli polymerase I (NEB #MO209S)
 1 mM Biotin-16-dUTP or DIG-11-dUTP (Roche #11093070910 or #11573152910)

Human Cot-1 DNA (Life Technologies #15279-011)

Salmon Sperm DNA (Life Technologies #AM9680)

3 M NaOAc

CEP Hybridization Buffer (Abbott Molecular #06L44-01)

Pre-hybridization solution

Denaturation solution

ADR001 Detection reagent (Cytocell Inc. #ADR-001)

VECTAShield with DAPI (Vector Labs #H-1200)

Mouse monoclonal α -digoxin-FITC conjugate

Streptavidin-Cy3 conjugate

STM solution

FISH detection solution

Prepare slides containing formalin fixed tissues

1. Bake slides overnight at 65°C
2. Let slides cool to room temperature
3. 3 changes of xylenes for 5 minutes each
4. 2 changes of 100% ethanol for 5 minutes each
5. Dry slides
6. Boil slides in microwave for 30 minutes on high with 10 mM citrate buffer pH 6.0, adding more buffer every 5 minutes to ensure tissue remains covered with liquid.
7. Let slides cool to room temperature on the bench top for about 30 minutes.
8. Encircle tissue with hydrophobic pen and continue to hybridization of slides.

Prepare biotinylated probe by nick translation

- 9 In thermal cycler tube add:

10 μ l	10X NT buffer
10 μ l	0.1 M β -mercaptoethanol
10 μ l	10X nucleotide stock
2 μ l	<i>E. Coli</i> polymerase
5 μ l	DNase I dilution
2.5 μ l	0.4 mM dTTP
4 μ l	1 mM Biotin-16-dUTP or DIG-11-dUTP
1 μ g	template DNA

add sterile water to 100 μ l

Using Biotin-16-dUTP will visualize red signal, while use of DIG-11-dUTP results in green signal.

- 10** Incubate 2 hr at 15°C then denature at 70°C for 10 minutes using thermal cycler.

- 11** Run 4 μ l of the reaction on a 1% agarose gel.

Expect fragments in the 300–600 bp range. If fragments are larger than 1000 bp, reincubate reaction with ~1.5–2.0 μ l DNase I dilution for 15–30 minutes at room temperature.

- 12** Inactivate enzyme by denaturing at 70°C for 10 minutes.

You can stop here and store the nick translated probe at –20°C.

Precipitate labelled probe

- 13** To each 20 μ l of labelled probe add (enough for 3–4 slides):

20 μ l labelled probe

4 μ g Human Cot-1 DNA

6 μ g Salmon sperm DNA

ddH₂O to 60 μ l total volume

6 μ l 3 M NaOAc

165 μ l cold ethanol

- 14** Centrifuge at 14000 RPM for 20 minutes at room temperature. Remove supernatant and allow pellet to air dry.

You can stop here and store dry pellets at –20°C.

Denaturation of probe

- 15** Resolubilize pellet in CEP hybridization buffer in 10–13 μ l (for 3–4 samples).

- 16** Denature probe using thermal cycler as follows:

70°C for 10 minutes

4°C for 5 minutes

Hold at 37°C until ready to use (>30 minutes)

Hybridization of slides

- 17** Set up moist incubation chamber in 37°C oven.

- 18** Pour pre-hybridization solution into Coplin jar and warm in 37°C water bath.

- 19** Pre-incubate an empty glass staining jar in 50°C water bath.

- 20** Place denaturation solution in 72°C water bath while it is warming.

- 21 Fill three Coplin jars with ethanols and hold them in the freezer just before step 23.
- 22 Place FISH slides into pre-hyb that has been warmed at 37°C. Incubate slides for 30 minutes.
- At this point the probe can be denatured, see above.
- 23 Remove ethanols from the freezer. Dehydrate each slide for 2 min in 70%, 80% and 95% ethanols. Discard the used 70% and refill the jar with fresh, but reuse the 80% and 95% for step 26.
- The procedure can be stopped here, slides air-dried and stored at -20°C
- 24 Pre-incubate the slides in the empty glass jar for 5 minutes in the 50°C water bath.
- 25 Place slides into denaturation solution for 2 minutes.
- 26 Repeat step 23 (ethanol series).
- 27 Dry slides and move them to the warming box until probe is prepared.
- 28 Apply mixed probe to slides (3 µl/sample).
- 29 Coverslip slides (using 1 cm round coverslips), avoiding bubbles.
- 30 Seal coverslip with parafilm.
- 31 Place slides into moist incubation chamber and incubate at 37°C overnight.

Detection of probe

- 32 Remove parafilm and coverslips from slides.
- 33 To remove unbound probe, wash the slides in 50% formamide, 2X SSC for 30 min at 50°C (25 mL formamide, 5 mL 20X SSC, 20 mL water).
- 34 Repeat step 33.
- 35 Wash the slides in 2X SSC for 30 min at 50°C (5 mL 20X SSC, 45 mL water).
- 36 Repeat step 35.
- 37 Use a 200 µl pipette tip to apply 1 drop ADR001 Detection reagent to tissue and cover with a tiny square of parafilm.
- 38 Incubate in the dark for 20 minutes at 37°C in moist warming box.
- 39 Wash twice in 4X SSC, 0.05% triton-X100 for 5 minutes at room temperature.
- 40 Apply 1 drop of VECTAShield with DAPI and coverslip.
- 41 Incubate for 10 minutes in the dark.
- 42 Detect the signal by a fluorescent scope.
- 43 For short-term storage (~ 1 week), seal edges of the coverslip with nail polish and store in a dark place.

For long-term storage, there is no need to add nail polish, just store at -20°C .

Reagents and solutions

10X F12 media:

- one packet (1 L) Ham's F12 Nutrient mix
- 90 mls ddH₂O
- 1.176 g NaHCO₃
- pH to 7.2
- bring to final volume of 100 ml with ddH₂O
- filter sterilize and store in 10 ml aliquots at -20°C

Fibroblast media:

- Ham's F12 Nutrient mix
- 10% FBS
- 1X Pen/Strep

Keratinocyte plating media (1.88 mM Ca²⁺):

- One part DMEM
- Three parts Ham's F12 Nutrient mix
- 0.5% FBS
- 1X hydrocortisone
- 1X cholera toxin
- 1X insulin
- 1X adenine
- 1X Pen/Strep
- 305 μl 1 M CaCl₂ for every 250 ml media

Cornification media 1 (1.88 mM Ca²⁺):

- One part DMEM
- Three parts Ham's F12 Nutrient mix
- 5% FBS
- 1X hydrocortisone
- 1X cholera toxin
- 1X insulin
- 1X adenine

1X Pen/Strep

305 µl 1 M CaCl₂ for every 250 ml media

Cornification media 2 (1.88 mM Ca²⁺):

One part DMEM

Three parts Ham's F12 Nutrient mix

2.5% FBS

1X hydrocortisone

1X cholera toxin

1X insulin

1X adenine

1X Pen/Strep

305 µl 1 M CaCl₂ for every 250 ml media

1000X 1,2-dioctanoyl-*sn*-glycerol, [C8:0] (10 mM):

50 mg 1,2-dioctanoyl-*sn*-glycerol

14.5 ml DMSO

filter sterilize and store in 1 ml aliquots at -20°C

1000X 5-bromo-2'-deoxyuridine, [BrdU] (10mM):

61.42 mg 5-bromo-2'-deoxyuridine

20 ml 1X PBS

filter sterilize and store in 1 ml aliquots at -20°C

100X hydrocortisone:

Dissolve a 25 mg vial in 5 ml cold 100% ethanol to make a 5 mg/ml solution (10000X).

This solution can be stored at -20°C indefinitely.

800 µl of 10000X stock from above

95 ml HBES

5 ml FBS

filter sterilize and store in 10 ml aliquots at -20°C, good for 6 months to a year

100X cholera toxin:

Add 1.2 ml sterile water to 1 mg vial, making a 10 µM solution.

dilute 100 µl of 10 µM solution from above into 100 ml HBES

add 0.1 g BSA

filter sterilize and store in 10 ml aliquots at 4°C, good for 6 months to a year

100X insulin:

12.5 mg	insulin
25 ml	0.005 M HCl

pre-wet filter with FBS and sterilize, store at 4°C, good for 2 weeks

100X adenine:

Dissolve 121 mg adenine in 50 ml 0.05 M HCl with stirring for 1 hour.

Filter sterilize and store in 10 ml aliquots at -20°C, good for 6 months to a year

HBES (HEPES buffered Earle Salts):

25 ml	1 M HEPES buffer
100 ml	10X Earle's Salts

bring to final volume of 1 L with sterile water and filter sterilize, store at 4°C

10X Earle's Salts:

NaCl	128 g
KCl	8 g
NaHCO ₃	74 g
NaH ₂ PO ₄ •H ₂ O	2.5 g
MgSO ₄ •7H ₂ O	4 g
Fe(NO ₃) ₃ •9H ₂ O	0.002 g
Phenol red	0.1 g

bring to 2 liters with ddH₂O
filter sterilize and store tightly capped at room temperature

3% H₂O₂:

5 ml	30% H ₂ O ₂
45 ml	methanol

10 mM citrate buffer pH 6.0:

10 ml	1 M citrate buffer pH 6.0
990 ml	ddH ₂ O

1 M citrate buffer pH 6.0:

29.41 g	sodium citrate, di-hydrate
90 ml	ddH ₂ O

adjust pH to 6.0
bring to final volume of 100 ml, filter and store at 4°C

2000X Hoescht's Dye:

1 mg	Hoescht's Dye
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10 ml ddH₂O
filter and store at 4°C

Anti-fade mounting medium:

dissolve 100 mg *p*-phenylenediamine in 10 ml 1X PBS, stirring vigorously at room temperature

adjust pH to 9.0

add 90 ml glycerol

store 10 ml aliquots at −380°C indefinitely, or at −20°C for a few months. When solution becomes dark, discard.

10X NT buffer:

0.5 M Tris-HCl pH 8.0

50 mM MgCl₂

0.5 mg/ml BSA

10X nucleotide stock:

0.5 mM dATP/dGTP/dCTP

DNase I stock solution:

0.15 M NaCl

50% glycerol

Dissolve DNase I into stock solution (20 U/ml final) and store at −20°C

Make a **1:1000 dilution** in ice cold ddH₂O immediately before use.

0.1 M β-mercaptoethanol:

Dilute 3.5 μl concentrated β-mercaptoethanol into 496.49 μl ddH₂O.

Pre-hybridization solution:

2X SSC

0.5% NP-40

Adjust pH to 7.0

Denaturation solution: (**Prepare fresh!**)

28 ml formamide

4 ml 20X SSC (**pH 5.3**)

8 ml ddH₂O

STM solution:

8 ml ddH₂O

0.5 g non-fat dried milk
2 ml 20X SSC
25 µl 20% Tween-20
2 µl 10% sodium azide

Combine water, 20X SSC and Tween-20 in appropriate sized tube.

Add non-fat dried milk.

Invert tube to mix well until no solids remain.

Filter sterilize using 0.8 µm filter, then 0.2 µm filter in series.

Add 2 µl of 10% sodium azide solution.

FISH detection solution:

Mouse monoclonal α -digoxin-FITC conjugate (2% by volume)

Streptavidin-Cy3 conjugate (1% by volume)

STM solution (97% by volume)

Add STM solution, α -digoxin-FITC conjugate and Streptavidin-Cy3 conjugate in appropriately sized tube.

Mix well.

Aliquot into amber 1.5 ml tubes, or wrap clear tubes with foil.

Store at 4°C in the dark.

Commentary

Background Information

NIKS (Near-diploid Immortalized Keratinocytes) are a spontaneously immortalized human foreskin keratinocyte (HFK) cell line isolated on the University of Wisconsin – Madison campus by Lynn Allen-Hoffmann (Allen-Hoffman et al., 2000). Karyotyping showed these cells harbor a duplication of the long arm of chromosome 8. When placed into an organotypic epithelial tissue culture system, these cells stratify and differentiate like normal HFKs and are able to recapitulate the full viral life cycle of human papillomaviruses (HPVs) (Flores et al., 1999). Sequencing of the p53 gene verifies it has the wild-type sequence (Allen-Hoffmann et al., 2000) and both p53 and pRb pathways appear to be intact (Flores et al., 1999, Lorenz et al., 2013). For these reasons, our lab routinely uses these cells to study the viral life cycle of human papillomaviruses.

An organotypic epithelial tissue culture, or raft, is a three-dimensional culture system used to mimic the architecture of stratified epithelia. Cells are grown submerged in media on a dermal equivalent composed of collagen and human fibroblasts. After four days, these cultures are lifted to the air/liquid interface and allowed to stratify and undergo normal differentiation for an additional 11 days. On day 11, rafts are incubated with BrdU reagent for eight hours, embedded in a solution of 2% agarose and 1% formalin, and allowed to solidify overnight. The following day, rafts are placed into histology cassettes and fixed in

10% buffered formalin overnight. Once fixed, the samples are placed into 70% ethanol and processed by our histology department to generate paraffin embedded samples that are then cut on a microtome. Slides containing 4 μ m sections of rafts are returned and analyzed for the following phenotypes: (See Anticipated Results)

Critical parameters and troubleshooting

Raft culturing:

- The recipe for the dermal equivalents listed in protocol is enough for the generation of 6 rafts. Scale up or down as necessary.
- When mixing the collagen pre-mix for the dermal equivalent, and again after the addition of the human fibroblasts, it's important to avoid generating bubbles. These bubbles could interfere with the architecture of the resulting epithelia and with feeding of the raft once lifted.
- Keep the collagen and collagen solutions on ice prior to plating in transwells. Collagen solidifies at room temperature and with increases in pH.
- If gels haven't solidified by 30 minutes, incubate longer or place in the incubator at 37°C, 5% CO₂. It's important that the dermal equivalents solidify.
- Dermal equivalents are ready to be used after 4 days. Keratinocytes should be plated before day 7.
- Be careful when aspirating the media from the inner transwell! It's very easy to damage or lose the dermal equivalent by aspiration.
- It's difficult to ascertain whether keratinocytes are growing on the dermal equivalent after lifting. Sometimes a shiny skin-like surface will appear indicative of a keratinized layer.
- It is important to keep the agar/formalin mix molten, but cool enough as to not destroy the raft when embedded. If the solution is too hot, cells could slough off the top of the raft with the addition. Maintain between 55°C and 65°C.
- Avoid air bubbles in the agar/formalin mixture as these bubbles will solidify and interfere when sectioning. They cause the microtome blades to dull prematurely.

BrdU staining:

- Once tissue is hydrated, never allow tissue section to dry out. This will result in an overall non-specific brown staining.

- H₂O₂ treatment will help decrease background staining due to endogenous peroxide.
- There are many types of antigen retrieval methods available. This protocol is optimized for BrdU staining, but other methods may give better results for different antigens. It is the responsibility of the researcher to discover optimal conditions for a given antigen.
- The 2 N HCl treatment is important for BrdU staining as this helps to denature the DNA and make the incorporated BrdU more accessible by the antibody. This step is not necessary for protein detection.
- A PAP pen or hydrophobic marker is not necessary, but it does help keep the hybridization solution localized to the tissue and allows one to use much less antibody solution per slide.
- To help decrease background, BSA and/or milk can be added to the blocking solution.
- All incubations should be performed in a humidified chamber to prevent drying out of the sample. Our lab uses moist paper towels inside a microscope slide box as our humidified chamber.
- DAB development should be monitored closely. It is impossible to undo an overdevelopment. In that sense, immunofluorescence is an easier process.

FISH:

- If biotinylated probe is not of appropriate size, and DNaseI treatment is repeated, it is very important to denature the enzyme at 70°C again. Otherwise, the researcher will be left with degraded probe.
- 20 µl of labeled probe (pre-precipitation) is sufficient for the hybridization of 3–4 slides. Scale up or down as necessary.
- Glass staining jars crack when taken from –20°C to water bath. It is suggested to use plastic for this purpose.

Anticipated Results

Unscheduled DNA Synthesis—Bromo-deoxyuridine (BrdU) is a nucleotide analog that is incorporated into newly synthesized DNA. In normal epithelia, or cells that stratify and differentiate normally in an organotypic culture, DNA synthesis is strictly limited to basal cells, which are those cells directly attached to the basement membrane. Upon cell division, daughter cells that lose contact with the basement membrane seed the suprabasal compartment of the stratified epithelia and undergo terminal differentiation. Once they lose

contact with the basement membrane, they enter a quiescent state (i.e. they do not re-enter S phase). When wild-type HPV is present in cells either through infection or transfection, the normally quiescent cells of the suprabasal layers incorporate BrdU, indicating they retain the ability to enter S phase. We refer to this as unscheduled DNA synthesis, and this is largely the result of E7's ability to inactivate pRb and pocket protein family members. HPV genomes lacking an intact E7 or expressing mutant forms of E7 that are defective in binding to pRb, p107 and p130 do not display this phenotype (Flores et al., 2000, Collins et al., 2005). An example of BrdU immunohistochemistry can be seen in Figure 2.

E7 Expression—In our lab, Mcm7 is used as a surrogate marker for the detection of E7 in an organotypic culture. We have been unable to detect physiological levels of E7 by immunohistochemistry (IHC) or immunofluorescence (IF) with currently available reagents. Through E7's binding to pRb, E2Fs are activated, which, in turn, upregulate transcription of Mcm7 (Brake et al., 2003).

In normal epithelia, or an organotypic raft of normal cells, Mcm7 expression is limited to the cells directly in contact with the basement membrane. When E7 is expressed, Mcm7 expression is expanded into the suprabasal cells, sometimes seen in the entire thickness of the epithelia or raft. See Figure 2 for Mcm7 immunofluorescence.

Differentiation Marker Staining—The Lambert lab uses the following differentiation markers to analyze organotypic rafts: keratin 10 (K10), involucrin and loricrin. In a normal, stratifying epithelia or raft culture, these protein markers are differentially expressed. Keratin 10 is normally detected in suprabasal cells, while involucrin is expressed in the granular layer along with loricrin. When cells are rafted that have been either infected or transfected with HPV, aberrant differentiation is observed. A dysregulation of the cell cycle results in a delay of the expression of keratin 10, and as a result, the other differentiation markers. Figure 2 illustrates a representative pattern of K10 and loricrin in an HPV-negative versus HPV16-positive raft culture of NIKS. Note, while keratin 14 is normally found to be restricted in its expression to basal cells in the epidermis *in vivo*, it is detected throughout all layers of raft cultures of NIKS (data not shown).

Late Stage of Viral Life Cycle—E1E4 is a late protein of HPV expressed in stratified cells. It is a marker used for the onset of the productive phase of the viral life cycle (Nakahara et al., 2005). Figure 2 illustrates a representative pattern of E1E4 expression in an HPV16-positive raft culture of NIKS. The E1E4 antibody was generated by John Doorbar and is detected by immunofluorescence either directly or indirectly.

Viral DNA Amplification by Fluorescence *in situ* Hybridization (FISH)—HPV DNA is amplified in the terminally differentiating compartment of stratified epithelia. This amplification is another hallmark of the productive phase of the viral life cycle and can be directly visualized in raft cultures of HPV-positive NIKS through the use of HPV-specific *in situ* hybridization. While our initial approach made use of radioactively labeled *in situ* hybridization probes (Flores et al., 1999), we have now modified our protocol to use fluorescence *in situ* hybridization (FISH) as described below and illustrated in Figure 2.

Capsid Formation—The HPV major capsid protein, L1, is another stain that can be performed to verify the productive phase of the viral life cycle (Flores et al., 1999, Flores et al., 2000). These viral capsids can also be seen by electron microscopy (EM) in raft cultures of cells harboring HPV (Flores et al., 1999, Genter Williams et al., 2005).

Time Considerations—Organotypic epithelial culture is a time consuming process. Not only does the experiment itself last for 16 days, but the preparation beforehand takes 4–7 days. In total, the duration of the experiment is 20–23 days. If the researcher decides to undertake this type of experiment they should plan for three weeks time for completion. While the day-to-day maintenance of the experiment is not time consuming, it will require their attention every other day. One should set aside 2 hours to generate the dermal equivalent, 3 hours for the plating of the keratinocytes, 30 minutes to lift the rafts and 9 hours for the BrdU labeling. Harvesting rafts will take between 30 minutes to an hour depending upon the number of rafts. Feedings can usually be accomplished in 30 minutes or less.

Immunohistochemistry—BrdU staining can be completed in two days. Most of the immunohistochemical stains performed in our lab call for an overnight incubation of primary antibody. We also perform our antibody incubations at 4°C which tends to result in lower background levels. If desired, this incubation could be done at room temperature or 37°C for a shorter duration of time.

FISH, like immunohistochemistry, is a 2-day process, consisting of an overnight hybridization step. This hybridization step is performed at 37°C.

Acknowledgments

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Figure 1. Harvesting rafts for histology

After taking a transwell insert out of a deep-well plate, remove the upper part of a transwell insert (A). Carefully lift a raft off of the transwell membrane (B), then place a raft on top of fixation solution (C). Add fixation solution on top of a raft to be embedded (D), then keep it overnight at 4°C.

Figure 2. Biomarkers of HPV life cycle in raft cultures

Shown are representative images of sections from rafts of HPV-negative (left column) and HPV16-positive (right column) NIKS stained with antibodies to indicated antigens or subjected to FISH (color of font indicates color of chromophore/fluorophore). In all cases of immunofluorescence (all except top row) sections were counterstained with DAPI (blue) to detect nuclei.