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Human histocultures (tissue explants) in retrovirology

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Summary

Viral pathogenesis is studied predominantly in cultures of primary isolated cells or cell lines. Many retroviruses efficiently replicate only in activated cells. Therefore, in order to become efficient viral producers cells should be artificially activated, a procedure which significantly changes cell physiology. However, for many viral diseases, like HIV-1 and other retroviruses' diseases, critical pathogenic events occur in tissues and cell isolation from their native microenvironment prevents single cell cultures from faithfully reflecting important aspects of cell-cell and cell-pathogen interactions that occur in the context of complex tissue cytoarchitecture. Tissue explants (histocultures) that retain tissue cytoarchitecture and many aspects of cell-cell interactions more faithfully represent *in vivo* tissue features. Human histocultures constitute an adequate model for studying viral pathogenesis under controlled laboratory conditions. Protocols for various human histocultures as applied to study retroviral pathogenesis, in particular of HIV-1, have been refined by our laboratory and are described in the present publication. Human histocultures of human tonsils and lymph nodes, as well as of recto-sigmoid and cervico-vaginal tissues can be used to study viral transmission, pathogenesis and as a pre-clinical platform for antivirals evaluation.

Keywords

Human tissue; HIV; culture; explants; pathogenesis; flow cytometry

1. Introduction

To study viruses, they first need to be isolated. From the very first virus, tobacco mosaic virus (VTM) isolated by a Russian scientist Ivanovsky(1), virus isolation represented the main challenge for virologists. Moreover, even the most advanced analytical methods of molecular biology as a rule require more than one viral particle. This is why the development of cultures of primary isolated cells or cell lines in the 1940's and 50's provided an indispensable source for viruses' isolation and for studying viral pathogenesis. Many retroviruses efficiently replicate only in activated cells. Therefore, to become efficient viral producers cell lines should be constitutively activated while primary cell cultures need

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to be activated. For example, PBMC used to produce Human Immunodeficiency Virus-1 (HIV-1) need to be activated and stimulated by incubation with an exogenous activator such as phytohemagglutinin (PHA) and IL-2, which may significantly change cell physiology. While such activation transforms cell cultures into efficient virus producers, for many viral diseases, like HIV-1 and other retroviruses' diseases, critical pathogenic events occur in tissues and the culture of isolated cells does not faithfully represent the *in vivo* situation. Even the very first steps in processing cells for cultures, namely tissue disruption and isolation of cells from their native microenvironment, prevent this system from faithfully reflecting important aspects of cell-cell and cell-pathogen interactions that occur in the context of complex tissue cytoarchitecture.

The field requires a model for the study of disease pathogenesis in tissues under controlled laboratory conditions especially because the closest animal model requires expensive non-human primates, whose adequacy is sometimes questioned anyway (2). Tissue explants (histocultures) that retain tissue cytoarchitecture and many aspects of cell-cell interactions more faithfully represent *in vivo* tissue features and thus serve an adequate model for studying viral pathogenesis.

Tissue explant models for studying various biological processes have a long history. Ross Harrison, an American biologist, is credited with the first successful work on artificial tissue culture in the early 1900's (3, 4). Shortly after, Alexis Carrel established cultures of chick embryo heart fragments, which he was able to maintain in culture for three months, thereby laying down the tenets of three dimensional tissue culture or "histoculture" (5). In the early 1950's Joseph Leighton improved upon Carrel's tissue culture system and introduced the idea of a sponge matrix as a substrate upon which to culture tissue (6, 7). In the 1980's Robert Hoffman utilized and improved upon Leighton's three dimensional histoculture method in his anticancer drug studies (8).

Other approaches to three-dimensional tissue culture have been developed. Nandi and colleagues (9) introduced the concept of culturing tissues embedded in collagen gels, Strangeways (10) and Fell and Robinson (11) developed strategies of organ culture on filter supports, Browning and Trier (12) recognized the importance of keeping cultures at the air liquid interface and developed rocking culture chambers, and Sutherland and colleagues (13) developed "spheroid" cultures with aggregation of cells in spinner flasks. Many other variations and techniques have since been developed each with their own advantages and disadvantages.

The technique of histoculture as applied to the study of microbial pathogens *ex vivo*, in particular retroviral pathogenesis, has been further refined by our laboratory. Human tissues used to study retroviruses, in particular HIV-1 include human tonsils and lymph nodes, and later recto-sigmoid and cervico-vaginal tissues (14–23).

Our *ex vivo* system of culturing human lymphoid tissue provides an important bridge between conventional two-dimensional cell culture and *in vivo* studies. While it has some limitations, some of which are shared by other experimental models, such as donor variability, inability to model effects of systemic factors, limited culture length (tissues

deteriorate after 3 weeks in culture), and difficulty in monitoring cells beyond the depth of confocal microscopy, this system has an important advantage: it allows investigation of normal and pathogenic processes, including those caused by human infectious agents, in the context of tissue cytoarchitecture under controlled laboratory conditions.

This system of human tissues *ex vivo* to study the pathogenesis of HIV supports productive HIV-1 infection without exogenous activation and stimulation, and retains tissue cytoarchitecture as well as the pattern of expression of key cell surface molecules relevant to HIV-1 infection (17, 24, 25). Also, some of the tissue functions are preserved *ex vivo*. For example, upon challenge with (recall) antigens such as diphtheria toxoid or tetanus toxoid, *ex vivo* lymphoid tissue responds with a vigorous production of specific antibodies (26) and cervico-vaginal tissue continues to produce mucus (27). *Ex vivo* human tissues also support replication of other viruses including human herpes virus (HHV)-6, HHV-7, human cytomegalovirus (HCMV or HHV-5), HSV-2 (HHV-2), vaccinia, measles virus, and West Nile Virus as well as pathogens such as the bacterium *Borrelia burgdorferi* and the parasite *Toxoplasma gondii*. Some of these organisms are commonly associated with HIV-1 and thus allow the simultaneous study of HIV and its copathogens. Below we present a detailed protocol for studying HIV-1 in human tissue *ex vivo*.

As the most available lymphoid tissue, we commonly use tonsils removed during routine surgery, but lymph nodes are also used for studies on HIV-1 pathogenesis. We also use cervico-vaginal and recto-sigmoid tissues as an *in vitro* model for viral transmission through sexual intercourse as these sites constitute the major port of HIV-1 entry. The *ex vivo* tissues can be used for pre-clinical evaluations of potential antivirals, and a procedure for using this system as a platform to test such compounds is described below as well. Although different tasks require different readouts, the most common final analysis is flow cytometry. The procedure to isolate cells from the tissues and analyze them constitutes the last section of the present paper.

Materials and Methods are detailed below for the following procedures

- Human lymphoid histocultures (blocks of tissue on collagen rafts) for studying HIV-1 pathogenesis (Chapter 3.1)
- Human cervical tissue for studying HIV transmission and early stages of infection (Chapter 3.2)
- Human histocultures as a platform for antivirals testing (Chapter 3.3)
- Tissue separation into single cells and analysis by flow cytometry (Chapter 3.4)

2. Materials

Prepare all solutions with ultrapure water and tissue culture grade reagents. Prepare and store reagents at room temperature unless otherwise indicated. Follow waste disposal regulations when disposing of biohazardous and chemical waste materials.

2.1 Histoculture components

1. Culture medium for the experiment: Prepare RPMI 1640 with L glutamine, MEM-nonessential amino acids, 10 mM (100×), MEM sodium-pyruvate, 100 mM (100×), Gentamicin (50 mg/ml, 1,000×), Fungizone: (250 µg/ml, amphotericin B, 100×), Fetal Bovine Serum (FBS) (*see* Note 1). Open a fresh 500-ml bottle of RPMI 1640, remove 90 ml (set aside for other use), and add 75 ml of FBS, 5 ml of 100× MEM-nonessential amino acids, 5 ml of 100× Fungizone, 5 ml of 100× MEM-sodium pyruvate, and 500 µl of 1000× Gentamicin. Store medium at 4°C, bring to 37°C before use on cultures.
2. Timentin, GlaxoSmithKline: Prepare 100X stock of Timentin, by adding one vial (3.1g) to 100 ml of DI water, freeze 1–2ml aliquots into screw-cap tubes at –30°C.
3. Culture medium for the initial incubation: This culture medium is to be used only for the first night of culture. Dilute 1 ml of freshly thawed 100× Timentin in 100 ml of explant culture medium as prepared above.
4. Collagen rafts: Gelfoam 12–7 mm (adsorbable gelatin sponge) Pharmacia and Upjohn Company, distributed by Pfizer (NY, NY). Calculate the number of Gelfoam pieces needed per experiment on the basis of the type of tissue used and the number of wells required to perform the experiment: 1.5 pieces per 6-well plate (tonsils, lymph node); 2 pieces per 12-well plate (cervicovaginal tissue); 3 pieces for one 24-well plate (rectosigmoid tissue).

2.2 Flow cytometry components

1. Digestion buffer: Prepare a 10-mg/ml solution by dissolving 1g of collagenase type IV: Gibco Invitrogen, into 100 ml of RPMI1640 supplemented with 5% FCS. Filter-sterilize through a 0.20-µm filter cap bottle. Distribute 1-ml aliquots into screw-cap tubes and keep frozen at –80°C.
2. FACS staining buffer: Prepare a staining buffer by adding 5 ml of normal mouse serum (NMS), Gemini Bioproducts and 5 ml of normal goat serum (NGS), Gemini Bioproducts to 1× PBS, pH 7.4 (1% final concentration, each). Filter-sterilize through a 0.2-µm filter-cap bottle.
3. Flow cytometry compensation beads, BD Biosciences for mouse and rat κ-chain-bearing antibodies respectively or Spherotech for mouse κ-chain-bearing antibodies): Prepare as many 12×75 tubes as there are fluorochrome conjugates used for cell staining. To do so, add a drop (≈40 µl) of anti-mouse κ chain (or anti-rat κ chain for rat monoclonal antibodies) and a prepare a tube containing a drop of control microsphere (*see* Note 2).

¹For culture optimization we usually test several lots of serum and select the lot that gives the highest viral replication. Then use the same lot of FBS for an entire series of experiments. In the case of viral replication, we always test several lots on tissues from several donors. Also, FBS is critical for the ability of tissue to secrete cytokines in culture medium: we observed that different lots differ in their ability to support secretion of various cytokines upon stimulation of tissues with several polyclonal activators. Another general problem is contamination that comes naturally with tonsillar tissues, or with intestinal tissues, which are not sterile by nature, or from the pathologists who evaluate tissue after surgery under non-sterile conditions. We use a cocktail of antibiotics to protect tissues from infection and change them depending on the evolving contaminations.

4. Dimethyl sulfoxide (DMSO), Sigma
5. Live-Dead fixable blue fluorescent reactive dye reagent kit, Invitrogen, or equivalent depending on available channels. Reconstitute according to the manufacturers' instructions by addition of 50 μ l of DMSO per dye vial (25 μ g). Prepare working stock of dye immediately before use by adding 1–2 μ l of dye to 1ml 1x PBS.
6. Paraformaldehyde, Electron Microscopy Sciences: Prepare 2% paraformaldehyde solution in 1X PBS.
7. Amine-modified particles, Bangs Laboratories or equivalent: Dilute the amine-modified stock solution into 1X PBS to a concentration of 10×10^6 beads/ml. Transfer an aliquot of 50 μ l into a 12×75 FACS tube and add 1 μ l of Live-Dead blue fixable dye. Incubate at room temperature in the dark for 20 minutes. Wash with 1 ml of PBS, centrifuge at 9,000 rpm for 3 minutes, decant the supernatant, add 100 μ l of PBS, vortex, add 100 μ l of 2% paraformaldehyde solution in PBS, and let stand for at least 20 minutes.
8. Fix & Perm: permeabilization reagent, Caltag-Invitrogen.
9. Cytometer Setup and Tracking beads (CST) beads, BD Biosciences
10. CountBright Absolute Counting Beads, Invitrogen
11. Anti-p24-PE-labeled antibody (KC-57), Coulter

3. Methods

3.1. Human lymphoid histocultures (blocks of tissue on collagen rafts) for studying HIV-1 pathogenesis (see Note 3)

The protocol described is designed for tonsillar tissue, probably the most available human lymphoid tissue, but with slight amendments (mentioned below) can be used for lymph nodes and rectosigmoid tissue. This procedure outlines infection with HIV, but can also be used for other pathogens and for co-infections (*see* Note 4). Perform procedures in biosafety cabinet and when working with pathogens follow appropriate biosafety precautions.

²If several conjugates of the same tandem dye are used, prepare a tube for each conjugate, as the spectral properties of each tandem dye may vary between antibodies and between lots of the same conjugate.

³One of the most important requisites for long-term culture of tissue is its good condition when delivered to the laboratory. In part, this condition depends on how quickly the tissues are delivered for processing. However, not all tissues are equal and some are more prone to deterioration than the others. Tonsillar tissue should be delivered within 3–5 hours post-surgery to avoid deterioration. Cervico-vaginal tissue is much sturdier, and we successfully culture this tissue delivered 24 hours post-surgery. The same is true of the rectosigmoid tissue that we used. Conditions of transportation also may be important. We use RPMI as a delivery medium; however, other solutions can be used. We have not optimized this step yet. We noticed that at least for tonsillar tissue cooling on ice during delivery makes it less suitable for long-term culture, we therefore have it delivered at room temperature, although we have not performed a systematic study of this issue as well.

⁴Human lymphoid tissues and tonsils in particular can be successfully infected by direct deposit of viral suspension on top of each tissue block. Single infections and HIV-1 co-infections with HIV-2, HSV-1, HSV-2, VZV, hCMV, HHV-6, HHV-7, Vaccinia virus, Measles virus, or West Nile virus have already successfully been performed in our laboratory. In addition non-viral pathogens can also be studied, and studied have been performed in our laboratory on *Borrelia burgdorferi* and *Toxoplasma gondii*. Co-infections are performed in a similar manner to single infections, one virus/pathogen is deposited on top of each tissue block, followed by deposition of the second pathogen.

1. Prepare Gelfoam sponges (*see* Note 5). Calculate the number of Gelfoam pieces needed per experiment as described in Materials. Depending on the number of pieces needed, fill a 100 × 20mm petri dish with 30 ml of culture medium or fill a 150 × 25mm petri dish with 50ml culture medium and add the Gelfoam sponges to the medium. Gently push the Gelfoam pieces into the medium using ethanol-sterilized forceps (*see* Note 6) to begin rehydration. Finish the hydration process by pressing on the Gelfoam with the bent flat spatula sterilized with ethanol. This also chases the air trapped in the sponge.
2. Use ethanol-sterilized scissors to cut the rehydrated Gelfoam into pieces of the appropriate size: Place a piece of Gelfoam into each well and add culture medium. 3 ml per well of a 6-well plate; 1 ml per well of 12-well plate; 0.5 ml per well of 24-well plate. Place the plates into a 37°C, 5% CO₂ incubator with high relative humidity (>83%) while tissues are being dissected.
3. Begin lymphoid tissue dissection and culture establishment (*see* Note 7). Wash tissues in PBS and transfer into a 100-mm petri dish containing 10–20 ml of PBS. Cut each tonsil into several large pieces (4–5 pieces per tonsil).
4. Transfer one piece into the lid of a new petri dish filled with 5 ml of medium, just enough to cover the lid. Cover the petri dish containing the rest of the tissues to avoid desiccation.
5. With a 23-gauge blade scalpel and forceps, remove the cauterized tissue from the tonsil, and remove the capsule surrounding the tissue. (If using lymph nodes, accurately remove fat). Remove bloody or inflamed parts, if any, and any parts containing tonsilloliths or parts with a suspicious color bordering on the light brown green.
6. Cut the tissues into slices of approximately 2-mm thickness. Remove any undesirable tissue, and re-cut the slices into strips of 2-mm width. Then cut the strips into 1-mm blocks. You should have a size of roughly 2×2×1 mm. Cut as many blocks as needed; at least 27 blocks are required per experimental condition. In case of using biopsy samples the number of replicate tissue blocks is limited by the IRB approval of the number of biopsies taken from one patient.
7. Transfer the blocks into a clean petri dish containing medium. Swirl the plate to randomize the distribution of the blocks. Take out the 6-well plate(s) containing Gelfoam from the incubator and, in each well, dispose 9 blocks of tissue on top of

⁵In the course of the development of explant technology, researchers have used different supports (rafts) to culture tissue on. These include filter paper, millipore filters, wire meshes, etc. In our experience as well as in the experience of other labs, the best-tested support is collagen gel such as one used for surgery. We use the product Gelfoam manufactured by *Upjohn*.

⁶Dry Gelfoam sponge is extremely brittle and might break apart if too much force is applied. Be extremely careful in pressing before rehydration starts. Also, the Gelfoam should be as free of air as possible: the presence of air will block the capillaries through which nutrients reach the tissue.

⁷The size of the blocks should be approximately 2×2×1 mm. Smaller blocks are more difficult to prepare, and also many cells are lost during cutting, which inevitably involves compressing and squeezing of the tissue. Larger blocks are easier to cut, but they cannot be cultured for long because of necrosis at their center, developing from the deficit of nutrients in the middle of the block. It is important for reproducibility of the results that tissues should be dissected into blocks of uniform size. We do it by hand with a blade or scalpel, and it can take several months for a researcher or a technician to become trained in cutting tissues into regular blocks. Our attempts to use various mechanical devices for cutting did not produce satisfactory results.

the Gelfoam, positioning each of them on its larger surface to maximize the contact area between the tissue and the Gelfoam.

8. Return the plate(s) to the incubator for overnight culture or proceed with infection. Keep the medium in which the dissection was performed as well as any unused piece(s) of tissue: they might be useful for an initial flow-cytometric analysis or for archiving DNA and RNA.
9. Begin infection of lymphoid tissue *ex vivo* with HIV-1. Tissues are infected by direct deposit of viral suspension on top of each tissue block. If the tissues were dissected the night before, aspirate the culture medium with a pipette.
10. Then tilt the plate and gently push the Gelfoam back to the high part of each well to allow the medium to gather at the lower part. Aspirate and discard this medium.
11. Add 3 ml of fresh medium to each well.
12. Dilute viral stocks to an appropriate concentration. It is preferable to have a dilution such that the desired inoculum is contained in 5 μ l of medium or less (*see* Note 8).
13. Pipet 5 μ l and delicately deposit this volume on top of each block. Let the plate stand under the hood for at least 15 minutes, and return it to the incubator.
14. Tissue is cultured for 12–14 days; samples of medium are collected periodically and analyzed for HIV-1 components and/or various metabolites of interest, in particular for cytokines (*see* Note 9). To sample, take two aliquots of medium from each of the replicate wells and distribute them into two labeled cryotubes. Repeat for all the experimental conditions.
15. Change medium every three days. With a pipette, aspirate the entire medium from the well, proceeding one experimental condition at a time. Keep the same pipette for the next step. Tilt the tissue culture plate, gently bring the Gelfoam to the top of the well, and let the medium flow down to the bottom; aspirate. Take a new pipette and add an adequate volume of fresh medium.
16. At the end of the experiment or at various time-points during it, tissue blocks are removed and dispersed into single cells for flow cytometry as described below. Also, samples of tissues can be fixed and analyzed immunohistologically.

⁸While it may seem reasonable to increase the volume of inoculum applied to the tissue, this is not a good idea since volumes larger than 5 μ l do not remain on the tissue block and end up diluted in the culture medium shortly after being applied.

⁹Empirically, we chose a 3-day period between consecutive medium changes. When the collected medium is analyzed for viral components (in the case of HIV-1 either for p24, RT or RNA) or for production of cytokines, it should be taken into account that after each medium change, the production of the compound of interest begins at a level of almost zero and reaches the value measured in the medium collected 3 days later. Formally, it should be presented as a “saw”-like graph. Therefore, we think that it is more adequate to present the release of components as a cumulative curve. Unlike cell lines or tissues from genetically identical mice, samples obtained from humans are quite variable in most parameters. For example, after inoculation with the same inoculum of the same HIV stock, HIV replication in tissues from different donors can differ by a factor of 20. Therefore, to compare results with tissues from different donors, it is useful to normalize parameters in the experimental group to the corresponding parameter in control tissue and then to average the results for tissues from different donors.

3.2 Human cervical tissue for studying HIV transmission and early stages of infection

Cervical tissue is the only tissue we have worked with thus far that requires different procedures for dissecting and infecting the tissue blocks; as with other tissues it can be used for co-infection studies as well (*see* Note 10).

1. Prepare Gelfoam sponges for cervicovaginal tissue as described in steps 1–2 for Section 3.1.
2. Begin cervico-vaginal tissue dissection and culture establishment. The tissue obtained after the surgery should be of a good quality: lacking large blood clots. Put the tissue into 100-mm Petri Dish containing 20 mL of complete culture medium with timentin.
3. With a 23-gauge blade scalpel and forceps carefully separate the ecto- and endocervix from the muscular tissue, leaving behind epithelium and underlying stroma (*see* Note 11).
4. Transfer the pieces of ecto- and endocervix into the lid of a Petri dish filled with 5mL of complete culture medium, cut them into uniform 2 mm × 2 mm × 2 mm blocks.
5. Swirl the blocks in order to randomize their distribution.
6. Remove the medium from the Petri dish lid with the pipette.
7. Count the blocks and determine how many experimental conditions are needed for your experiment (considering 16 blocks per condition for cervical tissue).
8. Begin infection of cervico-vaginal tissue *ex vivo* with HIV-1. Put the number of blocks (16 per condition for cervical tissue) to be infected into 1.5-ml Eppendorf conical tube, containing 0.5 ml of viral stock diluted appropriately.
9. Place the tubes in 37 °C pre-warmed Thermomixer with 300 r.p.m. continuous mixing.
10. Incubate for 2.5 hours.
11. Wash the blocks in a 6-well plate filled with 3ml of PBS: Put the blocks into wells, swirl gently with pipette tip, aspirate the PBS carefully without touching the blocks.
12. Repeat washing 3 times.
13. Take the 12 well plate with Gelfoam rafts from incubator and put 8 blocks on top of each piece of Gelfoam,
14. Return the plate (with tissue blocks) to the incubator and keep until the first medium change.

¹⁰Human cervicovaginal tissues are preferably infected by soaking tissue blocks in viral suspensions. HIV-1 co-infections with HSV-2 or hCMV have already successfully been performed in our laboratory.

¹¹Endocervix constitutes the lining of the lumen and usually has a darker color and is coated with thick mucus. The ectocervix constitutes the vault of the vaginal cavity and has a clearer color.

15. Three days after the infection, take two aliquots of medium from each of the wells and distribute them into pre-labeled cryotubes. Repeat for all the experimental conditions.
16. Aspirate medium from the wells.
17. Add 1 ml of culture medium into each well.
18. Perform sampling and change of medium every three days until 12 days after infection.
19. Store the samples at -80°C , until ready to measure viral replication by the appropriate assay.

3.3 Human histocultures as a platform for antivirals testing

Our procedure for testing of antivirals on histocultures involves overnight pretreatment of the cultures with the drugs followed by infection with virus the next day including fresh application of the drug on this day and on each medium change thereafter, except in the case of cervico-vaginal tissue where the antivirals are introduced at the time of infection with no pretreatment.

1. Prepare Gelfoam sponges for tissue as described in steps 1–2 for Section 3.1.
2. Dissect and prepare human tissue explants as described in Section 3.1 for lymphoid tissue or Section 3.2 for cervicovaginal tissue.
3. If using lymphoid tissue as a platform for testing antivirals complete steps 4–13, then skip to step 20 and complete procedure. For cervicovaginal tissue skip to step 14 and then complete procedure.
4. Prepare drug stocks by diluting the drug of interest in appropriate diluent for the drug.
5. Prepare a minimum of 30 μL of serial dilutions of the drug of interest 300 times more concentrated than the desired final concentrations (solutions 300X) by diluting the drug in appropriate drug-specific buffer.
6. In each well of a 6-wells plate containing 3 mL of complete medium supplemented with timentin, place nine blocks of tissue on top of the gelfoam, positioning each of them on its largest surface to maximize the contact area between the tissue and the gelfoam. A minimum of 3 wells per condition is required for statistical significance.
7. In each well containing 3 mL of medium, add 10 μL of each 300-times concentrated serial dilution of the drug of interest prepared as described in (5) so that the final concentration reached in each well is 1X. Slowly add the drug in the medium but not on top of the blocks. Once diluted, the medium may be delicately pipetted onto the block and gelfoam to ensure a faster diffusion. (28) In each drug-untreated control condition, add 10 μL of the drug specific buffer used to dilute the drug in step 5.
8. Return the plate(s) to the incubator for overnight incubation.

9. Begin infection of lymphoid tissue *ex vivo* 12 to 24h later. Aspirate the culture medium with a pipette. Note that the same pipette can be used to aspirate medium if starting with control and then experimental conditions with increasing concentrations of the drug of interest.
10. Add 3 mL of fresh complete medium to each well.
11. Add 10 μ L of each 300X serial dilution of the drug of interest per well as described in step 7.
12. Delicately deposit 5 μ L of viral stock on top of each block. Keep the plate undisturbed under the hood for 15 minutes.
13. Return the plate to the incubator until the next medium change (3 days later) where the same protocol of medium aspiration and drug treatment will be repeated.
14. When using cervicovaginal tissue as a platform for testing antivirals prepare a minimum of 25 μ L of 100X serial dilutions of the drug of interest in appropriate diluent.
15. During infection (see section 3.2 for full description of *ex vivo* infection of cervicovaginal explants), add 5 μ L of the drug of interest in a 1.5 mL Eppendorf conical tube containing 16 cervicovaginal blocks and 500 μ L of the virus of interest.
16. Transfer the tubes to the 37°C pre-warmed Thermomixer and set mixing to 300 rpm for 2h incubation.
17. In a 12-well plate, add 10 μ L of 100X serial dilutions of the drug of interest per well containing 1 mL of complete medium and a piece of Gelfoam.
18. Wash cervicovaginal tissue blocks with PBS and transfer 8 blocks on top of each Gelfoam piece (see section 3.2 for full description) making sure to match the drug condition concentrations during infection to those of the well.
19. Return the plate to the incubator until the next medium change (3 days later) where the same protocol of medium aspiration and drug treatment will be repeated.
20. Determine type of analysis to perform for assessing the antivirals' efficiency. The eventual toxic effect of the drug of interest must be addressed by comparing cell counts in untreated versus treated tissue blocks (steps 21–26). The effect of any antiviral of interest in *ex vivo* human tissue explants can also be evaluated by comparing the replication a given virus (steps 27–28) and the number of infected cells (steps 29–30) in absence or presence of the drug.
21. The possible toxic effect of the antiviral of interest is addressed by evaluating the decrease of infected lymphocytes in infected treated tissue blocks compared to matched infected untreated tissue blocks. Absolute cell numbers, and thus cell depletion, can be determined by flow cytometry.
22. Digest tissue and prepare single-cell suspensions for flow cytometry (see 3.4 below for full description).

23. Prior to cell surface staining, add Countbright absolute counting beads to each tube.
24. Stain single-cell suspension using a combination of surface markers to identify the cells of interest.
25. For each condition, acquire at least 10000 beads on a BD LSR II instrument using DIVA software version 3.0 and analyze data with FlowJo software.
26. Evaluate the effect of the antiviral of interest by comparing cell numbers in treated tissue blocks compared to matched untreated tissue block.
27. For studying the effect of a drug of interest on the replication of HIV-1, we measure productive viral replication from measurement of HIV-1 p24gag accumulated in tissue culture medium, using a dynamic immunofluorescent cytometric bead assay (29). Three days after the infection and before each medium change, take an aliquot of medium from each of the three replicate wells and distribute them into one labeled cryotube. Repeat for all untreated control and treated experimental conditions. Samples are measured at the end of the culture period.
28. Viral replication can also be assessed by real time PCR. Extract RNA or DNA depending on virus and perform amplification and detection real time PCR sequence detection system (or equivalent) using appropriate primers, probes, universal master mix, and standards.
29. For studying the effect of a drug of interest on the number of infected lymphocytes, digest tissue blocks and prepare single-cell suspension for flow cytometry (see section 3.4 below for full description).
30. Cells isolated from control and infected tissue blocks on day 12 post-infection are stained for surface markers, washed two times, fixed and permeabilized with Fix&Perm, and then either stained with an anti-p24-PE-labeled antibody to identify HIV-1 infected cells if the drug of interest is tested for its anti HIV activity or stained with an antibody recognizing a viral antigen of the virus against which the drug is tested for its anti-viral effect.

3.4. Tissue separation into single cells and analysis by flow cytometry

1. Begin tissue digestion by picking up with forceps tissue blocks from the Gelfoam and transfer into a 1.7-ml Eppendorf tube containing 875 μ l of RPMI-5% FBS, DNaseI (0.2 mg/ml). Transfer up to 27 blocks of tonsil or lymph node, 8 rectosigmoid tissue biopsies or 16 cervicovaginal tissue blocks.
2. Add 125 μ l of collagenase IV (10 mg/ml).
3. Shake the tubes at 900 r.p.m for 1 hour in a Thermomixer set at 37 °C.
4. Place the strainer-cap pre-wetted with 1ml of PBS on top of 50 ml tube (one per condition) filled with 20 ml of PBS.
5. Transfer 500 μ l from digestion tube into appropriate strainer-cup.

6. Scar the lid of a small Petri dish in crossing series of parallel lines. Deposit tissue blocks, add PBS to wet the lid, and squeeze tissue blocks between the scarred lid bottom and the bottom of a syringe plunger.
7. Transfer content into the corresponding strainer-cup.
8. Rinse the lid with several ml of PBS; pass through the strainer.
9. Centrifuge at 400g for 5 min; discard the supernatant (*see* Note 12).
10. Resuspend the pellet in 1ml of PBS.
11. Begin flow cytometry staining by mixing a volume of approximately 50 μ l of cell suspension from each condition and keep them on ice (unstained tube).
12. Add 1 μ L of working stock of Live–Dead fixable blue stain solution to the cell suspension.
13. Incubate 15 min.
14. Add 2 ml of PBS and centrifuge at 400g for 5 min; discard the supernatant.
15. Resuspend the pellet in 50 μ l of PBS per staining condition.
16. Label the FACS tubes (1 per condition) and add 50 μ l of cell surface antibody mix.
17. Transfer 50 μ l of cell suspension from 50 ml tubes to FACS tubes containing antibody mixture.
18. Prepare the compensation tubes by adding one drop of flow cytometry compensation beads; add the same amount of each individual antibody used in the staining.
19. Incubate tubes prepared in points 17 and 18 for 20 min at room temperature in the dark.
20. Repeat point 14. If performing cell surface staining only, skip to step 30; if performing intracellular staining, continue with step 21 and complete the rest of the procedure.
21. Gently vortex the pellet to separate cell aggregates.
22. Add 100 μ l of Fix & Perm solution A to each tube, including compensation beads.
23. Incubate at room temperature for 15 min.
24. Add 2 ml of PBS and centrifuge at 400g for 5 min, discard the supernatant.
25. To the tubes containing cells, add 100 μ l of Fix & Perm solution B.
26. Add 30 μ l of intracellular staining antibody cocktail (previously titrated) and 15 μ l of NMS.
27. Incubate at room temperature for 20 min.

¹²To avoid exposure to aerosols when spinning cells to prepare single-cell suspension, use centrifuge buckets that can be sealed tightly. Load and unload the buckets under a biosafety cabinet.

28. Repeat step 24.
29. Re-suspend in 100 μ l of PBS; vortex gently.
30. Add 100 μ l of 2% (wt/vol) paraformaldehyde solution in PBS; vortex.
31. Incubate at room temperature for 1 h (to inactivate virus).
32. Keep samples in the dark at 4 °C and acquire samples on flow cytometer within 48 h. Set up the flow cytometer with adequate quality control using cytometer setup and tracking beads or equivalent if you are not running DIVA (*see* Note 13).
33. Run and record the appropriate compensation controls and unstained control.

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References

1. Sebastian, A. A Dictionary of the History of Science. Informa Healthcare; Pearl River, NY: 2001.
2. Brown CR, Czapiga M, Kabat J, Dang Q, Ourmanov I, Nishimura Y, Martin MA, Hirsch VM. Unique pathology in simian immunodeficiency virus-infected rapid progressor macaques is consistent with a pathogenesis distinct from that of classical AIDS. *Journal of virology*. 2007; 81:5594–5606. [PubMed: 17376901]
3. Harrison R. Observations on the living developing nerve fiber. *Anat Rec*. 1907; 1
4. Harrison R. The outgrowth of the nerve fiber as a mode of protoplasmic movement. *J Exp Zool*. 1910; 9:787–846.
5. Carrel A. On the permanent life of tissues outside of the organism. *J Exp Med*. 1912; 15:516–528. [PubMed: 19867545]
6. Leighton J. A sponge matrix method for tissue culture; formation of organized aggregates of cells in vitro. *J Natl Cancer Inst*. 1951; 12:545–561. [PubMed: 14889259]
7. Leighton J. A method for the comparison of the fate of intravascular tumor-cell emboli in vivo and in organ culture. *Natl Cancer Inst Monogr*. 1963; 11:157–195. [PubMed: 13929432]
8. Hoffman RM. Three-dimensional histoculture: origins and applications in cancer research. *Cancer Cells*. 1991; 3:86–92. [PubMed: 2054260]
9. Yang J, Richards J, Bowman P, Guzman R, Enami J, McCormick K, Hamamoto S, Pitelka D, Nandi S. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. *Proceedings of the National Academy of Sciences of the United States of America*. 1979; 76:3401–3405. [PubMed: 291014]
10. Strangeways, T. *Tissue Culture in Relation to Growth and Differentiation*. W Heffer and Sons Ltd; Cambridge, England: 1924.
11. Fell HB, Robison R. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro. *The Biochemical journal*. 1929; 23:767, 784, 765. [PubMed: 16744264]
12. Browning TH, Trier JS. Organ culture of mucosal biopsies of human small intestine. *The Journal of clinical investigation*. 1969; 48:1423–1432. [PubMed: 5796354]
13. Inch WR, McCredie JA, Sutherland RM. Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture. *Growth*. 1970; 34:271–282. [PubMed: 5471822]

¹³Before running samples on the flow cytometer, verify the proper function and setup of the flow cytometer lasers, fluidics, and optical channels using the cytometer setup and tracking beads if running DIVA 6.0. Alternatively, use similar approaches to ensure proper function and setup of the instrument. Detailed instructions are available in a Nature Protocol contributed by Perfetto et al.(30). A poorly set-up flow cytometer will give unreliable data. It is imperative that the instrument be set up and verified daily. Compensation settings and LASERs delays should be performed for each experiment.

14. Glushakova S, Baibakov B, Margolis LB, Zimmerberg J. Infection of human tonsil histocultures: a model for HIV pathogenesis. *Nat Med.* 1995; 1:1320–1322. [PubMed: 7489416]
15. Glushakova S, Baibakov B, Zimmerberg J, Margolis L. Experimental HIV infection of human lymphoid tissue: Correlation of CD4+ T cell depletion and virus syncytium-inducing/non-syncytium-inducing phenotype in histoculture inoculated with laboratory strains and patient isolates of HIV type 1, *AIDS Res. Retroviruses.* 1997; 13:461–471.
16. Grivel JC, Ito Y, Faga G, Santoro F, Shaheen F, Malnati MS, Fitzgerald W, Lusso P, Margolis L. Suppression of CCR5- but not CXCR4-tropic HIV-1 in lymphoid tissue by human herpesvirus 6. *Nat Med.* 2001; 7:1232–1235. [PubMed: 11689888]
17. Condack C, Grivel JC, Devaux P, Margolis L, Cattaneo R. Measles virus vaccine attenuation: suboptimal infection of lymphatic tissue and tropism alteration. *J Infect Dis.* 2007; 196:541–549. [PubMed: 17624839]
18. Grivel JC, Garcia M, Moss W, Margolis L. measles virus inhibits HIV-1 replication in human lymphoid tissue ex vivo. *J Inf Dis.* 2005 (in press).
19. Lisco A, Grivel JC, Biancotto A, Vanpouille C, Origgi F, Malnati MS, Schols D, Lusso P, Margolis LB. Viral interactions in human lymphoid tissue: Human herpesvirus 7 suppresses the replication of CCR5-tropic human immunodeficiency virus type 1 via CD4 modulation. *Journal of virology.* 2007; 81:708–717. [PubMed: 17065205]
20. Biancotto A, Iglehart SJ, Lisco A, Vanpouille C, Grivel JC, Lurain NS, Reichelderfer PS, Margolis LB. Upregulation of human cytomegalovirus by HIV type 1 in human lymphoid tissue ex vivo. *AIDS Res Hum Retroviruses.* 2008; 24:453–462. [PubMed: 18327985]
21. Lisco A, Vanpouille C, Tchesnokov EP, Grivel JC, Biancotto A, Brichacek B, Elliott J, Fromentin E, Shattock R, Anton P, Gorelick R, Balzarini J, McGuigan C, Derudas M, Gotte M, Schinazi RF, Margolis L. Acyclovir is activated into a HIV-1 reverse transcriptase inhibitor in herpesvirus-infected human tissues. *Cell host & microbe.* 2008; 4:260–270. [PubMed: 18779052]
22. Vanpouille C, Biancotto A, Lisco A, Brichacek B. Interactions between human immunodeficiency virus type 1 and vaccinia virus in human lymphoid tissue ex vivo. *Journal of virology.* 2007; 81:12458–12464. [PubMed: 17804502]
23. Saba E, Grivel JC, Vanpouille C, Brichacek B, Fitzgerald W, Margolis L, Lisco A. HIV-1 sexual transmission: early events of HIV-1 infection of human cervico-vaginal tissue in an optimized ex vivo model. *Mucosal Immunol.* 2010; 3:280–290. [PubMed: 20147895]
24. Fletcher PS, Elliott J, Grivel JC, Margolis L, Anton P, McGowan I, Shattock RJ. Ex vivo culture of human colorectal tissue for the evaluation of candidate microbicides. *Aids.* 2006; 20:1237–1245. [PubMed: 16816551]
25. Glushakova S, Yi Y, Grivel JC, Singh A, Schols D, De Clercq E, Collman RG, Margolis L. Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue ex vivo. *J Clin Invest.* 1999; 104:R7–R11. [PubMed: 10487781]
26. Glushakova S, Grivel JC, Fitzgerald W, Sylwester A, Zimmerberg J, Margolis LB. Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. *Nat Med.* 1998; 4:346–349. [PubMed: 9500611]
27. Boukari H, Brichacek B, Stratton P, Mahoney SF, Lifson JD, Margolis L, Nossal R. Movements of HIV-virions in human cervical mucus. *Biomacromolecules.* 2009; 10:2482–2488. [PubMed: 19711976]
28. Biancotto A, Grivel JC, Lisco A, Vanpouille C, Markham PD, Gallo RC, Margolis LB, Lusso P. Evolution of SIV toward RANTES resistance in macaques rapidly progressing to AIDS upon coinfection with HHV-6A. *Retrovirology.* 2009; 6:61. [PubMed: 19573243]
29. Biancotto A, Brichacek B, Chen SS, Fitzgerald W, Lisco A, Vanpouille C, Margolis L, Grivel JC. A highly sensitive and dynamic immunofluorescent cytometric bead assay for the detection of HIV-1 p24. *Journal of virological methods.* 2009; 157:98–101. [PubMed: 19100289]
30. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality assurance for polychromatic flow cytometry. *Nat Protoc.* 2006; 1:1522–1530. [PubMed: 17406444]