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# Human Lymphoid Histocultures

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## Summary

Human tissue explants provide a valuable tool for experimental studies of normal and pathological tissue physiology. They allow us to understand intercellular interactions by mirroring their original spatial relationship within body tissues, and can be employed to model host-pathogen interactions under controlled laboratory conditions. Here we describe a protocol to process and infect with human immunodeficiency virus (HIV) type 1 lymphoid tissue explants from human tonsils and maintain them in culture at the liquid-air interface in a non-polarized setting. These *ex vivo*-infected tissues have been successfully used to study the biology of early HIV-1 pathogenesis, as well as a platform to test the efficacy and toxicity of antiviral drugs.

### Keywords

Lymphoid tissue; tonsils; human tissue explants; collagen sponge; HIV-1; ex vivo culture

## 1. Introduction

Studies of human pathophysiology are restricted by the complexity of human bodies as well as by obvious ethical reasons. History of biomedical studies teaches us that experimental models are required for the identification of normal and pathological mechanisms. Since the introduction of these models into biomedicine at the beginning of the XX century the cultures of isolated cells became a standard system due to their availability, relative simplicity, and reproducibility of experimental settings and results. To these conventional cultures we owe the most outstanding accomplishments in cellular and molecular biology. On the other hand, such monotypic two-dimensional cell cultures do not account for the spatial and functional communication between the large variety of cell types that compose tissues and organs. This aspect is of paramount importance for experimental models of various diseases, as interference with homeostatic intercellular interactions is the leading factor of many pathologies.

For example the critical events of HIV infection take place in lymphoid tissue where this virus infects a small fraction of lymphocytes, but infection leads to the death of uninfected cells (“bystander death”) (1) and deterioration of the entire structure of lymphoid tissue (2). Paradoxically, tissue explant cultures (histocultures) preceded conventional cell cultures. Ross Harrison, an American biologist, performed the first successful work on artificial tissue culture in the early 1900s (3). Shortly after, Alexis Carrel successfully cultured chick

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embryo heart fragments for 3 months, thereby laying down the foundations of three-dimensional tissue culture (4). Later, researchers switched to single cells cultures and it took about 50 years before the next improvement of explant culture technique took place: Joseph Leighton introduced the idea of a sponge matrix as a substrate and then Robert Hoffman in the 1980s perfected the three-dimensional histoculture method in his anticancer drug studies (5).

In the middle of the1990s, our laboratory pioneered the technique of human lymphoid tissue culture, mostly for purposes of studying the pathogenesis of HIV (6), where lymphoid tissue is a major site of virus replication.

The system of lymphoid histoculture offers major advantages over single cell cultures as explants retain tissue cytoarchitecture and many important functional aspects of cell–cell interactions *in 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 (7). Like any other *ex vivo* model, human lymphoid tissue culture has its own limitations: these include donor variability, problems with tissue polarization, tissue survival that is limited to ~ 3 weeks, and difficulty in monitoring cells beyond the depth of confocal microscopy.

Nevertheless, human lymphoid tissue *ex vivo* remains a model of choice to study homeostatic and pathogenic immunological processes in humans, including host-pathogen interactions (reviewed in (8)). In particular, the culture of human lymphoid tissue allowed HIV-1 infection and transmission to be studied under controlled laboratory conditions, as upon inoculation *ex vivo*, lymphoid tissue explants support productive HIV infection without exogenous cell activation, and retain the pattern of expression of key cell surface molecules relevant to HIV infection (9, 10). The preservation of functional structures (e.g., follicules and germinal centers) within tissue explants offers a unique opportunity to integrate spatial and functional aspects of the infection, providing a potential insight into the mechanisms regulating viral persistence. *Ex vivo* lymphoid tissues were also successfully used for preclinical evaluations of potential antivirals (11–13). Here, we describe a detailed protocol for the culture of human lymphoid tissue obtained from tonsillar specimens, from tissue dissection to explant culture on top of collagen sponges, as well as infection of explants with HIV-1. This technique, unlike culture of tissues fully immersed in medium, provides more oxygen while delivering nutrients from the bottom through the sponge capillaries. Such a combination, together with the natural collagen substrate of the sponge, significantly delays necrosis of the center of the explants that is typical for immersed tissues.

## 2. Materials

### 2.1 Preparation of collagen sponges

1. Sterile collagen sponges. In the protocol we refer to Gelfoam® Absorbable Collagen Sponge USP, 12–7 mm format (Pfizer, NDC: 0009–0315-08) as an example (*see* **Note 1**).
2. Sterile Petri dish, 100 mm x 20 mm or wider.

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3. Culture medium (CM): RPMI1640 with L-glutamine and phenol red, 100μM Modified Eagle's medium (MEM)-nonessential amino acids (FisherThermoScientific), 1mM sodium-pyruvate, 50μg/ml gentamycin sulfate, 2.5μg/ml amphotericin B (FisherThermoScientific), 15% fetal bovine serum (FBS). We recommend testing different batches of FBS before purchasing a large stock (*see* **Note 2)**.
4. Sterile metal forceps or tweezers.
5. Sterile metal scissors.
6. Sterile flat weighing metal spatula.
7. 6-well culture plates.
8. Water-jacketed $CO_2$ incubator, set at 37°C, 5% $CO_2$, ≥90% humidity.
9. Water bath.

### 2.2 Processing of human tonsillar tissue

1. Tissue transportation medium: sterile phosphate buffer saline (PBS) or other solution with physiologic pH.
2. Sterile transportation container (*see* **Note 3**).
3. Specimen of human tonsils obtained from routine tonsillectomy or tonsillotomies (*see* **Note 4**).
4. Culture medium (CM).
5. Timentin solution (100x): add 100 ml of sterile water cell culture-grade to a 3.1 g vial of Timentin® (GlaxoSmithKline, NDC: 0029–6571-26) (*see* **Note 5**). Aliquot and store at −20°C.
6. Sterile Petri dish, 100 mm x 20 mm.
7. 70% ethanol solution
8. Disinfectant solution for biological waste disposal (see **Note 6**).
9. Sterile forceps or tweezers.
10. Sterile scalpels and blades (*see* **Note 7**).
11. Cell-free HIV-1 viral preparation(s) (*see* **Note 8)**.

## 3. Methods

All experiments are independently carried out using explants from the same tissue donor to set up experimental conditions (i.e donor-matched). The simplest experimental design comprises a minimum of 2 conditions: treated vs. untreated control (e.g. HIV-infected vs. uninfected tissue explants). We recommend including at least 2 technical replicates for each experimental condition. Calculate the number of tissue explants and collagen sponges

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required by each experiment in advance in order to minimize the waste of reagents while processing tissue specimens (Tab. 1).

Although it is not strictly necessary to prepare collagen sponges before tissue dissection, we recommend following the order outlined here especially when handling large amount of tissue for many experiments.

### 3.1 Preparation of collagen sponges.

1. Supplement CM with timentin solution (CMT) before use. Prepare enough CMT medium to dissect tonsillar tissue and culture tissue explants. Unused thawed timentin aliquots should be discarded. Unused medium containing timentin can be stored at 4°C for 2 to 4 weeks but it must be re-supplemented with timentin before use.
2. Fill a 100 mm x 20 mm-Petri dish with about 20–30 mL of CMT. This setting is optimal to prepare 3 Gelfoam® 12–7 mm sponges at the time. If larger amounts of sponges are required, the use of a wider Petri dish and larger CMT volume may be more convenient and speed up the preparation.
3. Transfer the collagen sponges into the Petri dish using sterile forceps and press the sponges against the bottom of the Petri dish for about 2 min using a sterile spatula (*see* **Note 9**).
4. Use sterile scissors to cut the rehydrated collagen sponges into 4 pieces of equal size.
5. Transfer 1 sponge piece using forceps into each well of a 6-well culture plate.
6. Add 3 mL of CMT into each well.
7. Place the plates in the incubator until tissue explants are ready to be loaded on the sponges.

### 3.2 Dissection of human tonsillar tissue

1. Allow CM enough time to reach room temperature (RT) or put it in a water bath pre-warmed at 22–37°C.
2. Supplement CM with timentin solution (CMT) before use.
3. Pour 70% ethanol solution into a clean container to soak and clean forceps and scalpel whenever needed during the dissection procedure. We recommend cleaning tools and changing scalpel blade in between handling specimens from different donors.
4. Transfer tonsils from the transportation medium into a 100 mm-Petri dish containing CMT using forceps. Specimens with widespread areas of cauterized, bloody or necrotic tissue should be discarded (*see* **Note 10**, Fig. 1).
5. The lid of the petri dish can be used as a cutting surface to dissect tissue. Holding the tissue gently with forceps, cut each tonsil into several big pieces using sterile scalpel and blade. Remove cauterized, bloody, and fibroid tissue, and any parts

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containing tonsilloliths (calcified material) and/or with green-brownish color. While working on one piece of tissue, it is important to keep the rest of the tissue submerged in medium to avoid tissue desiccation (*see* **Note 11**).

6. Cut a tissue piece into slices of about 2 mm in thickness. Remove any unwanted part as in the previous step. Cut the tissue slices into 2 mm-thick strips. Cut the tissue strips into 2 mm-thick blocks. This should result in tissue blocks of roughly 8 cubic mm, also referred to as explants (Fig. 2).
7. Transfer the explants into a new 100 mm-Petri dish containing CMT to avoid desiccation while proceeding with tissue dissection.
8. Swirl the plate to randomize tissue explant distribution.
9. Place 9 tissue explants on top of each collagen sponge in a 6-well plate using forceps allowing spacing between explants (Fig. 2).
10. Place the plates in the incubator (*see* **Note 12**).
11. Dispose of all biological waste in an apposite container containing disinfectant solution.

### 3.3 Infection of tissue explants.

If infection is not required, go to paragraph 3.4. Typically, explants are cultured overnight and inoculation of cultures with HIV-1 is performed the next day (see **Note 12**).

1. Put CM in a water bath pre-warmed at 37°C.
2. Supplement CM with timentin solution (CMT) before use. Unused thawed timentin aliquots should be discarded. Unused medium containing timentin can be stored at 4°C for 2 to 4 weeks but it must be re-supplemented with timentin before use.
3. Aspirate the medium in the six-well plate with a pipette and discard it in a container with disinfectant solution. Tilt the plate and gently push the collagen sponges to the upper part of the well to allow the medium to gather at the bottom, aspirate and discard it.
4. Add 3 mL of CMT to each well.
5. Thaw the HIV-1 viral preparation in the water bath pre-warmed at 37°C. If necessary, dilute viral stock to an appropriate concentration. It is preferable to have a dilution such that the desired inoculum is contained in a 5 to 8 μL-volume. It is critical for an efficient infection to allow the minimum time required between thawing HIV-1 viral preparation and infecting tissue explants.
6. Pipette the inoculum directly on top of each of the 9 blocks on a collagen sponge (*see* **Note 13**).
7. For an accurate delivery of the inoculum we suggest using a reverse pipetting technique and changing the pipet tip for every well. Return the plate to the incubator as soon as infection is completed.

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### 3.4 Culture of tissue explants

Change CM to the explant culture every three days starting from tissue dissection (or infection). Explant culture supernatant and/or explants may be harvested at regular intervals throughout culture time to monitor a number of parameters (*see* **Note 14**).

1. Put CM in a water bath pre-warmed at 37°C. It is not required to supplement CM with timentin.
2. Aspirate the medium in the six-well plate with a pipette and discard it in a container with disinfectant solution. Tilt the plate and gently push the collagen sponges to the upper part of the well to allow the medium to gather at the bottom, aspirate and discard it.
3. Add 3 mL of CM to each well.
4. Using forceps, put back on a sponge any blocks of tissue that may have fallen off from it.
5. Return the plate to the incubator.
6. Repeat steps 1 to 5 every three days until day 12–15 post-dissection or -infection (*see* **Note 15**).
7. Dispose of the plates with any residual medium, tissue explants and collagen sponges.

## 4. Notes

1. Collagen sponges are hemostatic devices prepared from purified porcine skin, which are meant to be completely absorbed by the human body after a few weeks. Similar products are available from a number of providers. We have chosen Gelfoam® due to its slow degradation rate in culture that better suits the purpose of maintaining tissue explants at the liquid-air interface for 2 weeks, compared to other collagen sponges that dissolve rapidly. Spongostan™ Standard, Absorbable Haemostatic Collagen Sponge 7×5×1 cm (Ferrosan Medical Devices, ref. MS0002) is a valid alternative that we tested and used in our experiments. In general, we recommend testing different batches of the same product in order to assess its performance in explant culture.
2. We advise testing several lots of FBS for culture optimization and use the same lot of FBS for an entire study. We routinely test FBS on tissue explants from 10 donors and select the lot that gives the highest HIV-1 replication. Of note, FBS can also affect the ability of tissue explants to secrete cytokines.
3. Pour enough liquid into the container so that the tonsils may stay submerged in it after surgery and during transportation to avoid tissue desiccation. We recommend leaving the specimens in transportation medium at room temperature.
4. The protocol for collection of human tissues requires ethical approval by the local competent authorities. Obtaining personal and medical data (e.g. reason for

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surgery, current drug use, history of infections, etc.) from tissue donors may provide important information to interpret experimental results, although it poses important concerns regarding informed consent and privacy that must be addressed in the protocol for tissue collection.

5. Timentin® is a mix of the antibiotics ticarcillin and clavulanate that are commercially available as individual reagents. These antibiotics efficiently prevent the growth of bacteria that, in our laboratory, often contaminate tissue samples. Each laboratory should determine which antibiotics work best for their purposes. For example, penicillin and streptomycin can be used instead of Timentin, although they have different properties. For example, Timentin displays low stability at room temperature or 37°C (about 24 hours) therefore, once added to culture medium, it remains active only for the first day of culture.

6. The entire procedure should be carried out in a biological safety cabinet. All human specimens, even those from 'healthy' individuals, may harbor infectious agents. The operator should receive training to work with blood-borne pathogens to safely handle human tonsillar tissue specimens, even if experiments do not involve the use of exogenous infectious agents (e.g. HIV). A risk assessment of the entire procedure should be carried out by trained staff at the laboratory to ensure the safety of the operator and coworkers.

7. Handling sharp tools to dissect human tissue specimens puts the operator at more risk of accidental injury and contamination. The operator should consider wearing metal mesh cut-resistant gloves as additional protection.

8. For most of our experiments we have used the following viral preparations: HIV-$1_{BaL}$ and HIV-$1_{LAI.04}$ obtained from the clarified culture medium of peripheral blood mononuclear cell cultures inoculated with either HIV-$1_{BaL}$ or HIV-$1_{LAI.04}$, originally received from the NIH AIDS Reagent Program. A Biosafety Level 3 facility, or dedicated BSL2 laboratories with BSL3 practices, and adequate training are required to handle infectious agents.

9. Collagen sponges are extremely brittle when dehydrated. The hydration process should be done carefully especially when pushing down on the foam with the spatula to chase the air out. When sponges are first added to medium, wet sponges on both sides then allow a few minutes for the sponges to begin soaking up medium before pushing down with any force. Fully hydrated sponges should be as free of air as possible: the presence of air will block the capillaries through which culture medium nutrients reach the tissue.

10. We recommend processing tonsils the same day of surgery. If that would not be possible, specimens may be left overnight submerged in culture medium containing antibiotics at 4°C. Take note of any feature of tissue specimens that you suspect may affect experimental results, such as bloody, cauterized and necrotic parts. We suggest saving an aliquot of medium in which tissue dissection is performed and/or a few tissue explants for measuring cell viability, nucleic acids extraction, and/or histological analysis.

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**11.** During dissection, take care to handle tissue gently as cells are easily released from tonsil tissue. Keeping a small volume of medium in the petri dish while sectioning a particular piece will prevent the tissue from floating around and making dissection more difficult. But be mindful to keep the rest of the tissue covered completely in medium. Scalpel blades should be changed often to prevent unnecessary damage to tissue with dull blades.

**12.** We prefer performing infection after an overnight incubation to make sure that no bacterial or fungal contamination develops. In addition, cells tend to egress tonsillar tissue blocks after dissection. This process is largely completed within the first 24 hours of culture.

**13.** In some circumstances, it may be preferable to infect by soaking tissue blocks in a small amount of medium mixed with virus (max volume of 500μL) in a 1.7ml microcentrifuge tube for 2 hours at 37°C. Tissue blocks can then either be transferred onto sponges, or washed first and then transferred. Although more laborious, this way of infecting tissue explants may give better result in tissue where HIV replication is not very high, such as in cervico-vaginal tissue (14).

**14.** Explant culture offers a number of possible experimental readouts. For nucleic acid extractions or histology tissue explants can be snap-frozen, preserved in apposite solutions for RNA stabilization, or embedded in formalin. For flow cytometry we immediately process tissue blocks by digesting them with an enzymatic cocktail to obtain a single-cell suspension for staining. Even if it is not required by the experiment, we recommend saving tissue explants for storage at −80°C once the culture is ended. Explant culture supernatant can be used to measure the secretion of soluble factors (e.g cytokines) or HIV-1 replication over time. Our standard read-out is to measure the concentration of the HIV-1 core protein $p24_{gag}$ in samples of culture supernatant collected every third day post-infection (15).

**15.** During culture, tissue outgrows into collagen sponges. Also individual cells may migrate out of the tissue into the sponges. One can analyze the part of tissue that is above the gel separately by pinching it out with forceps. The cells within the gel can be squeezed out using forceps or a syringe plunger. However, one should keep in mind that what is extracted from the gel is not just a fraction of migrated cells but rather a mixture of such cells and tissue structures.

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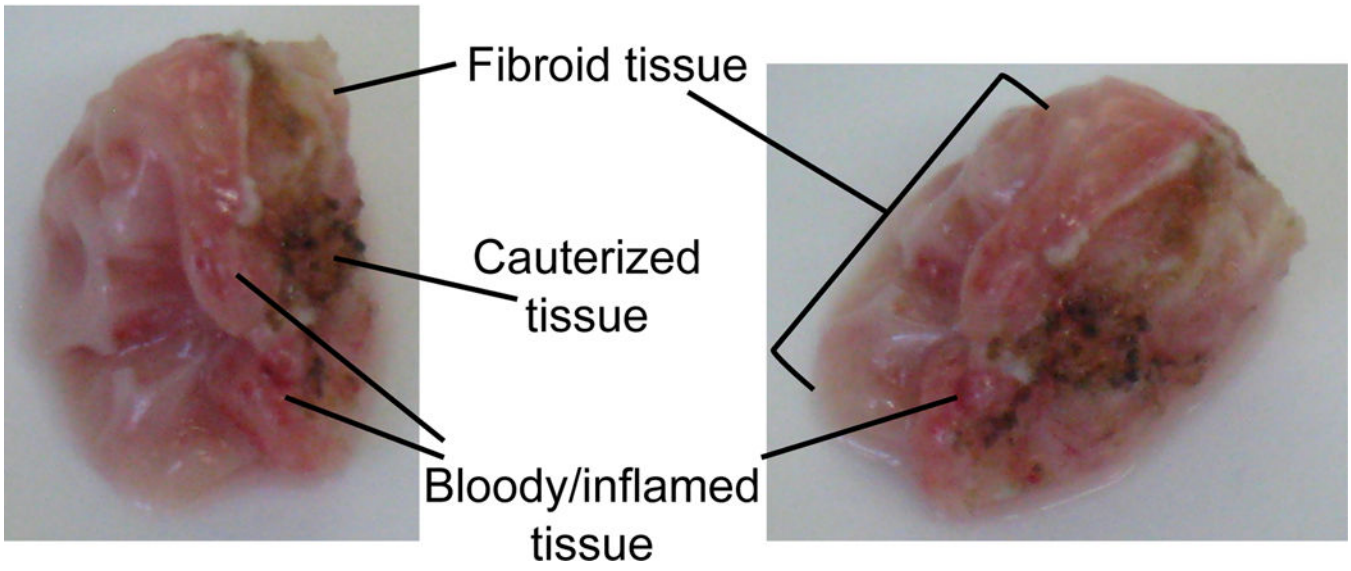


**Fig 1: Human tonsil histoculture preparation.**

Tonsils are obtained from tonsillectomy or tonsillotomy. Indicated are the areas that should be removed before proceeding with tissue dissection: residual fibroid tissue from the capsule surrounding the tonsil (white), cauterized tissue (brown-black), and bloody/inflamed tissue (red). Also, parts with brown-greenish color that is indicative of tissue necrosis, should be removed.

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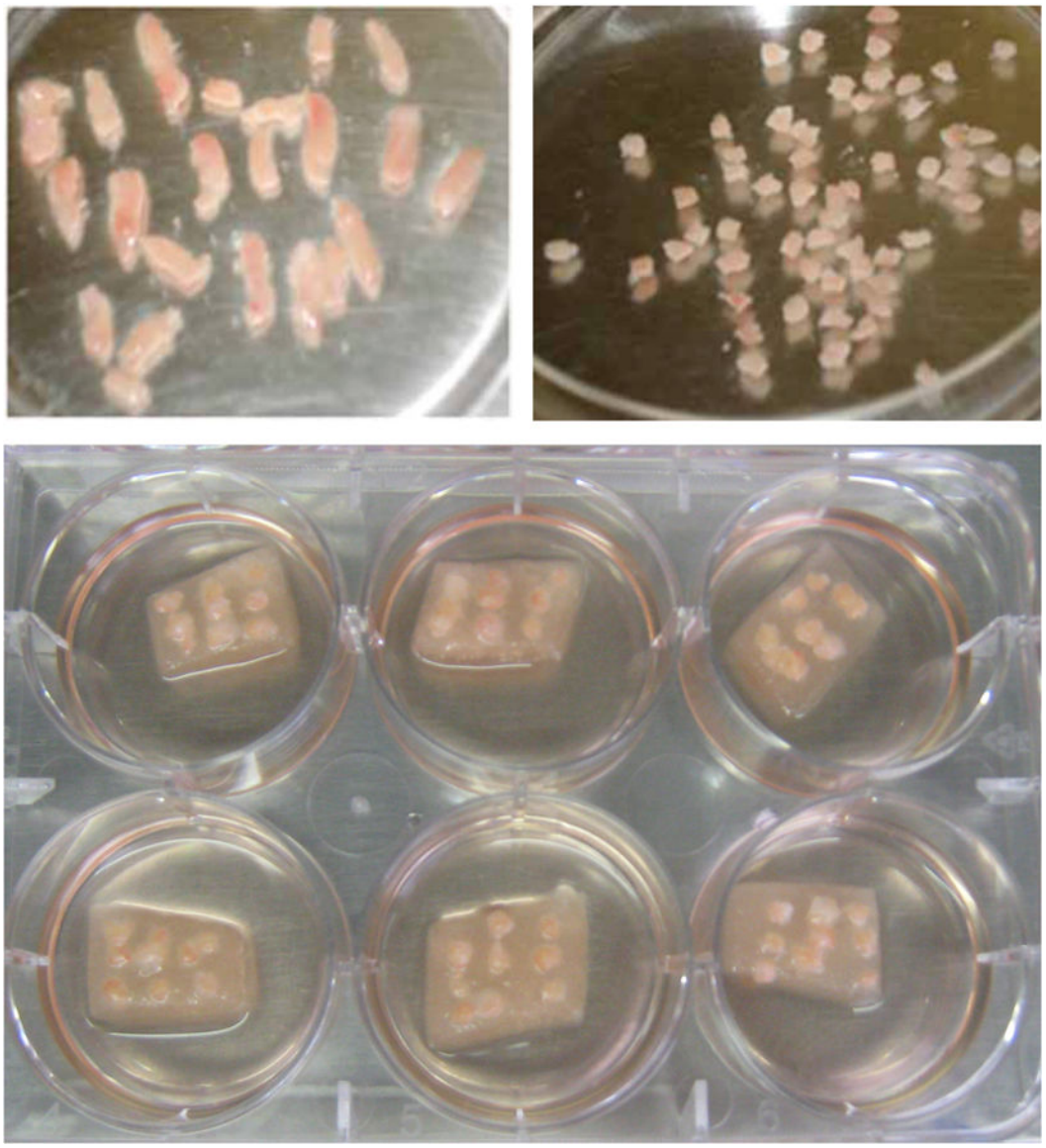

**Fig 2: Dissection and culture of human tonsillar tissue.**

Pieces of tonsils cleared from unwanted parts (e.g. fibroid, cauterized, bloody, necrotic as indicated in Figure 1) were dissected into strips (upper left) and next into blocks of approximately 8 cubic mm (upper right). Tonsillar tissue explants on top of collagen sponges (9 explants/sponge) in a 6-well plate containing culture medium (bottom).

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**Table 1.**

Human lymphoid histocultures

| Number of Collagen sponge pieces per 12–7 mm sponge | Volume of medium per well of 6-well plate (mL) | Number of blocks per a sponge piece (corresponding to one well) | Number of Replicates (wells) per Experimental condition |
|---|---|---|---|
| 4 | 3 | 9 | minimum: 2 |