Chapter 8

The Preparation of Chicken Tracheal Organ Cultures and Their Application for Ciliostasis Test, Growth Kinetics Studies, and Virus Propagation

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Abstract

Chicken tracheal organ cultures (TOCs) provide a simple ex vivo system that makes use of transverse section of tracheal rings extracted from embryos or adult birds to perform classical virological techniques for virus isolation, propagation and titrations, alongside with gene-expression analysis and virus-host interaction studies. Most IBV strains replicate well in TOCs, thus conveniently allowing growth kinetics analysis. Viral replication is revealed by observation of ciliostasis as marker of infection in tracheas extracted from birds ex vivo, as well as in vitro analysis providing a reliable infection model and a useful tool for titration.

Key words Tracheal organ cultures, Ciliostasis, Virus titration, Virus passaging, Respiratory virus

1 Introduction

Tracheal organ cultures (TOCs) are largely used in microbiology studies across multiple disciplines [1], finding their place in virology, bacteriology, parasitology, toxicology and immunology due to the mimicking of in vivo conditions applicable in in vitro experiments to investigate respiratory pathogens [2, 3]. Use of TOCs in avian virology is well established, having provided in vitro infection models to extensively study morphological and functional effects on the respiratory epithelium for avian influenza (IAV), Newcastle disease virus (NDV), avian metapneumoviruses (aMP), infectious laryngotracheitis virus (ILV), avian adenoviruses and, particularly, infectious bronchitis virus (IBV) [4–6]. TOCs allow for virus isolation, quantitative titrations and growth kinetic studies, providing a valid method for isolating field or recombinant viral strains, given the high sensitivity and a targeted set of cellular receptors. More recently, pathogenicity studies and host interaction analysis have been conducted using ex vivo TOCs, extending the use of TOCs in

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the field of vaccinology, as they offer a subsidiary method to test virus pathogenicity and assess virus protection [7–10].

In this chapter, we first describe the preparation of TOCs from tracheas of 19-day-old embryos, as those are reported to be equally susceptible as at 9-day-old [4]. However, the same protocol with specific modifications applies to adult TOCs when extracted from 2 to 3-week-old birds. Age of birds does slightly affect sensitivity when comparing the ciliostasis activity of IBVs at 3 days post infection (p.i.), however, use of adult TOCs allow for greater viral yield, as quantitatively more tissue is susceptible to virus replication, therefore, based on the purpose of the study, use of adult rather than embryo TOCs may be more appropriate. Cultivation of TOCs may be successful in multiwell plates [11], however use of a tissue culture rolling drum with associated racks is preferred as it reduces accumulation of debris preserving viability of the TOCs and allowing for clearer observation of the cilia beating. Following preparation of TOCs, protocols for ciliostasis tests, growth kinetics analysis and propagation of IBV in TOCs for passaging are provided in detail. Ciliostasis tests are commonly carried out during in vivo experiments to evaluate tracheas ex vivo to examine vaccine protection [10, 12] and correlation with virus pathogenicity [7]. Additionally, observation and scoring of ciliary activity following infection of TOCs in vitro correlates with virus infectivity and helps define virus characteristics, thus allowing further applications, such as the use of TOCs for titrations by determining the ciliostatic dose (CD50). Some IBV strains may be poorly ciliostatic, thus precluding the use of TOCs for titrations by scoring the cilia activity. Most IBV strains replicate in TOCs regardless of their ability to cause ciliostasis, therefore TOCs can still be used to investigate replication dynamics and for propagation and passaging of IBV to assess viral genetic stability. Following preparation of TOCs, screening and selection of TOCs suitable for use is fundamental before proceeding with any technique. TOCs must always be viable and clear of any bacterial contamination (see Note 1). TOCs work may be labour intensive but, at the same time, it represents an invaluable in vitro model of infection for IBV studies.

In this chapter, we describe updated protocols for preparation of embryonic TOCs, a modification of method described previously by Hennion [13]. We also include methods for preparation of TOCs from 2 to 3-week old chickens, and the application of TOCs for the study of viral growth kinetics, ciliostasis and virus propagation.
2 Materials

2.1 Preparation of Tracheal Section

1. 19- to 20-day-old embryonated eggs from specific pathogen free (SPF) chicken flock or tracheas extracted from 2 to 3-week-old chickens.
2. Tissue chopper e.g. a McIlwain mechanical tissue chopper (Mickle Laboratory Engineering Co. Ltd.).
3. Double-edged razor blades.
4. Sterile curved scissors (small).
5. Sterile scissors (large).
6. Sterile forceps.
7. Sterile scalpel.
8. Sterile Whatman filter paper discs 55 mm diameter (see Note 2).
9. 70% industrial methylated spirits (IMS).
10. Penicillin + streptomycin (100,000 U of each per ml).
11. 1 M HEPES buffer prepared from HEPES (free acid) and tissue culture grade water, sterilized in an autoclave at 115 °C for 20 min.
12. Culture medium: Modified Eagle’s Medium (MEM), 40 mM HEPES buffer, 250 U/ml penicillin, and 250 U/ml streptomycin.
13. Sterile Bijou bottles or similar.
14. Sterile 100- and 150-mm-diameter petri dishes.
15. Sterile phosphate buffered saline (PBS).
16. Large bore Pasteur pipette.

2.2 Culture of Tracheal Section and All Applications

1. Tissue culture roller drum capable of rolling at approximately 8 revolutions/h at 37 °C.
2. Associated rack suitable for holding 16 mm tubes on roller drum.
3. Sterile, extra-strong rimless soda glass tubes 150 mm long and 16 mm outside diameter, suitable for bacteriological work (see Note 3).
4. Sterile silicone rubber bungs 16 mm diameter at wide end, 13 mm diameter at narrow end, and 24 mm in length (see Note 4).
5. Inverted microscope.
3 Methods

To calculate the number of embryonated eggs required for an assay, assume that each trachea will yield 17–20 rings. For adult TOCs about the same number of rings is expected for a single trachea as the proportions are conserved and the rings are thicker. The same procedures apply in both cases with a few modifications specified in the protocol. Expect a loss of up to 20% of the TOCs during the preliminary incubation step, due to damage to the rings during preparation or spontaneous cessation of ciliary activity. Those TOCs that have reduced visibility of the lumen but are clearly viable need to be excluded from ciliostasis tests but may be included in other studies, for example growth kinetic analysis or passaging.

3.1 Preparation of Tracheal Sections

1. On a clean workbench spray or wipe the top of the eggs with 70% IMS (see Note 5).
2. Using curved scissors or forceps remove the top of the shell, lift the embryo out by the wing and cut off the yolk sac. Place the embryo in a 150 mm petri dish and discard the egg and yolk sac.
3. With a sharp pair of scissors decapitate the bird, severing the spinal cord just below the back of the head and angling the cut to just below the beak (see Note 6).
4. Position the embryo on its back and, using small forceps and scissors, cut the skin along the length of the body from the neck to the abdomen. Care must be taken not to damage the underlying structures.
5. Locate the trachea and using small scissors and forceps, dissect it away from the surrounding tissues (see Note 7).
6. Cut the trachea at the levels of the carina and larynx (the larynx may have been removed on decapitation) and remove it from the embryo, placing the tissue in a Bijou bottle containing culture medium or a petri dish (see Note 8).
7. Repeat steps 2–6 for all available embryos.
8. Place one trachea at a time on a disc of filter paper and, using two pairs of fine forceps, gently remove as much fat as possible (see Note 9).
9. For adult TOCs, as the size allows, wash them with PBS, flushing through with a Pasteur pipette.
10. Place the cleaned tracheas in a 100 mm petri dish containing culture medium.
11. Swab the tissue chopper with 70% IMS.
12. Place two filter paper discs on top of the plastic cutting table disc and slide the assembled discs under the cutting table clips on the tissue chopper.
13. Raise the chopping arm of the tissue chopper and attach the razor blade.

14. Position the arm over the center of the cutting table (see Note 10).

15. Place the tracheas on to the filter paper under, and perpendicular to, the raised blade and moisten with a small amount of culture medium (see Note 11).

16. Adjust the machine to cut sections 0.5–1.0 mm thick and activate the chopping arm. Do not forget to set the settings for embryo or adult TOCs, accordingly (see Note 12).

17. Once the arm has stopped moving, discard the first few rings from each end of the cut tracheas; then with a scalpel, scrape the remaining rings into a 150-mm petri dish containing culture medium.

18. With a large bore Pasteur pipette or similar gently aspirate the medium to disperse the cut tissue into individual rings (see Note 13).

19. Repeat steps 11–17 until all the tracheas have been sectioned (see Note 14).

### 3.2 Culture of Tracheal Sections

1. With a large bore Pasteur pipette or similar dispense one to five TOC rings into a glass tube previously filled with 1 ml culture medium (see Note 15).

2. Seal the tube with silicone bung or lid and check that each tube is filled with the exact number of rings selected (see Note 16).

3. Put the tubes in the roller tube rack, place on the roller apparatus and set to roll at approximately 8 revolutions/h, at approximately 37 °C. Leave the tubes rolling for 1–2 days (see Note 17).

### 3.3 TOC Assessment and Selection

1. Check each tube for complete TOC rings and the presence of ciliary activity, using a low power inverted microscope.

2. Discard any tubes in which less than 100% of the luminal surface has clearly visible ciliary activity.

3. For tubes containing more than one ring the luminal surface may not be fully accessible to inspection, in that case observing the approximate viability of the rings and carefully evaluating the medium for bacterial contamination will be sufficient.

### 3.4 Ciliostasis Test During In Vitro Infection

1. Prepare the viruses for the infections accordingly to the titre set in advance. The small volume for the infections (100–200 μl) may be a limiting factor to consider when deciding the infection titres.
2. For each virus select 10 replicates of 1×TOC tubes and arrange the tubes in rows on a standing rack. Include 10 tubes as negative control for mock infections and at least 1 positive control virus of known ciliary activity.

3. Wash the TOCs rings with PBS (see Note 18).

4. Inoculate each tube with 100–200 μl of virus, or with TOC medium for the mock infected tubes. Make sure the TOCs are immersed in the infection liquid.

5. Apply the lids or silicone bungs.

6. Mark the top of the lids or the tubes for identification.

7. Incubate the tubes at 37 °C for 1 h standing upright in the rack without rotation.

8. At the end of the incubation time top up with 900 μl of TOC culture medium and incubate on the rotor in the rolling drum at 37 °C, approximately 8 revolutions/h.

9. Starting at day 3 post-infection assess the ciliary activity of all the TOCs infected and the controls by light microscopy. Take note of the score until the ciliary activity is completely abrogated in the test samples. Ciliary activity is scored as 4, 3, 2, 1 or 0 corresponding to 100, 75, 50, 25 or 0% residual ciliary activity. Mock infected TOCs should retain close to 100% ciliary activity until the end of the experiment.

10. For most accurate results, the ciliostasis test should be repeated three times with the same controls and the average trend recorded.

### 3.5 Assessment of Ciliary Activity Ex Vivo

1. Tracheas extracted from chickens experimentally infected with IBV are collected in PBS during necropsy examinations and taken to the lab for processing.

2. Wash and flush the TOC rings through with PBS.

3. Remove the excess of tissues and fat using forceps and scalpel.

4. Arrange the trachea lengthwise and use a scalpel to cut four rings approximately 1–2 mm thick from the centre and three rings from each end. Place all the ten rings on a microscope slide keeping them hydrated with PBS.

5. Assess ciliary activity using the same scoring method as described in Subheading 3.4, step 9.

6. The same tissue can be re-used to assess presence of viral genome by RT-PCR following RNA extraction, or other non-sterile techniques.

### 3.6 Growth Kinetics Study

1. Determine the number of tubes required with the appropriate number of TOC rings per tube based on the time points selected and number of viruses to test, always including enough
control tubes for mock infection and ideally a control virus with known growth kinetics. Generally, we use three to five tubes, each containing three TOCs, per time point per virus.

2. Prepare the viruses for the infections at a known titre. As it is not easy to determine the multiplicity of infection (MOI) with TOCs, a higher/lower viral titre may be selected based on the purpose of the study.

3. Arrange the number of required TOCs tubes in rows on a standing rack (see Note 19).

4. Wash the TOC rings with PBS (see Note 18).

5. Remove any PBS and inoculate each tube containing TOCs with 500 μl of virus, or with TOC medium for the mock infected TOCs. Make sure the TOCs are immersed in the infection liquid.

6. Apply the lids or the silicone bungs to close the tubes.

7. Mark the top of the lids or the tubes for identification.

8. Incubate the tubes at 37 °C for 1 h standing upright in the rack without rotation.

9. At the end of the incubation time, wash the TOC rings with PBS twice.

10. Remove the final wash and add 1 ml of TOC medium per tube.

11. Incubate the tubes on the rotor in the rolling drum at 37 °C, approximately 8 revolutions/h.

12. At each time point collect the supernatants, and the TOCs if required, for titrations and further analysis.

### 3.7 Virus Propagation in TOCs

1. Determine the number of tubes required with the appropriate number of TOC rings per tube. Generally, we use 3 to 5 replicate tubes per virus, containing each 3 to 5 TOC rings, including a mock infected control and ideally, a control virus with a known ability to propagate in TOCs.

2. Prepare the viruses for the infections, diluting to a particular titre in TOC medium if required.

3. Arrange the number of required TOCs tubes in rows on a standing rack.

4. Wash the TOC rings with PBS (see Note 18).

5. Remove the PBS and inoculate each tube with 200–500 μl of virus/TOC medium per tube. Make sure the TOCs are immersed in the infection liquid.

6. Close the tubes by applying the lids or the silicone bungs.

7. Mark the top of the lids or the tubes for identification.

8. Incubate the tubes at 37 °C for 1–2 h standing upright in the rack without rotation.
9. At the end of the incubation time, wash the TOCs with PBS.
10. Remove the PBS and add 1 ml TOC medium per tube.
11. Incubate the tubes on the rotor on the roller drum at 37 °C, approximately 8 revolutions/h, for 24 h or longer, based on the individual growth kinetics of the selected virus strain.
12. Harvest the supernatant, and the TOC rings if required, and proceed with further passages following the same methods (see Note 20).

4 Notes

1. When assessing TOCs for ciliostasis tests, TOCs are selected if they display a very neat and clear lumen to allow further inspections at the light microscope. However, this criterion is not as strict when selecting TOCs for growth kinetics tests or virus propagation, as including several TOCs per tube prevents a clear observation of the whole lumen.
2. Batches of sterile Whatman filter papers can be prepared by interleaving individual discs with slips of grease-proof paper and placing them in a glass petri dish. Wrap the dish in aluminium foil or in paper bags and sterilize in a hot air oven (160 °C for 1 h) or autoclave (120 °C for 20 min).
3. Batches of sterile tubes can be prepared by placing them, open end down, in suitable sized lidded tins lined with aluminium foil or in paper bags. Sterilize in a hot air oven or autoclave as in Note 2.
4. Batches of sterile silicone rubber bungs can be prepared by placing them, narrow end down, in shallow, lidded tins. Sterilize by autoclaving at 120 °C for 20 min. Alternatively, plastic lids compatible with the tubes may be purchased for single use. In this case always be careful to fit the lids tightly and stabilise with some autoclave tape if required.
5. Preparation of TOCs can be performed on the open laboratory bench after cleaning the surfaces with 70% IMS or any other suitable disinfectant.
6. Care must be taken at this stage not to damage the trachea.
7. The trachea can be identified by the presence of transverse ridges seen down its length owing to the underlying rings of cartilage.
8. The carina and larynx can be identified by the increased diameter at either end of the trachea.
9. To avoid damage to the trachea hold it as close to one end as possible with the first pair of forceps and use the second pair to strip away the fatty tissue.
10. At this stage gently lower the arm on to the cutting area disc, loosen the screw holding the blade slightly, check that the blade is aligned correctly (the full length of the blade must be in contact with the cutting area), tighten the screw again, and raise the arm.

11. A maximum of five tracheas can be laid side by side on the cutting bed at any one time. Gently stretch each trachea as it is placed on the cutting area, and when all five are in the correct position, wet them with a few drops of culture medium.

12. Settings of the chopper require to be adjusted for TOCs extracted from embryos or adult birds. Using the McIlwain mechanical tissue chopper set the control knob at 2–3 for embryo TOCs, and 8 for adult TOCs.

13. TOCs prepared from adult birds cannot be separated through a Pasteur pipette as they are too large, instead use a pair of forceps to separate each individual ring and to place them in the tubes.

14. It is important to use a fresh blade and paper discs for each set of five tracheas to be sectioned and ensure used blades are disposed of in an appropriate sharps bin.

15. The number of rings that can be allocated per tube depend on the assay performed, however it also changes based on the TOC size. For growth kinetics, when more than one TOC per tube is required, we usually dispense 3–5 embryo TOCs or a maximum of 3 TOCs from 2 to 3 week old chickens. The total number of TOCs available should also be considered.

16. Check for damaged glass tubes at this stage, particularly around the rims. Discard any with cracks as these can fail when bungs are inserted, leading to injured fingers. Make sure the tracheal rings are fully submerged in culture medium and not stuck on the wall of the tube. Discard any that are ragged or incomplete. Check the lids fit perfectly and seal properly those that are loose and might come off during the rolling with tape.

17. Make sure that the drum is aligned correctly on the apparatus and that the roller is actually rotating before leaving the cultures to incubate; the speed of the roller apparatus is slow.

18. A quick and easy way to speed up the washing step is to add few ml of PBS without removing the media in the tube, then with a rapid and confident rotation of the hand pour it on a stack of tissues being careful not to lose the TOC ring. Finally, remove the excess of PBS by aspiration or using a pipette. This step is useful when taking into account that a large number of tubes need to be processed in the shortest time possible.
19. When multiple viruses are tested at the same time, we suggest working in a team so that the washing step and the addition of virus or medium are carried out in the quickest time for all the samples.

20. The volume generated through passaging is limited, so take it into consideration when planning for further assays. The volume of medium can be reduced to increase the titre and replicates may be pooled together to increase the volume available.

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