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623.2.HTC_Precision_Cut_Lung_Slices V.2

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We use this protocol and it's working

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Abstract

Purpose and Scope of the Procedure or Laboratory Assay

1. Provide high quality, viable, 500 μ m sections of human lung for culture and other in vitro assays
2. Current preparation has limited ability to produce quality sections with larger cartilaginous airways or sections containing certain pathological features (ex. metaplastic bone).

Protocol Version 2 is modified to reflect the use of Precisionary Instruments Compresstome® VF-510-0Z vibratome for thick sectioning the agarose inflated tissue blocks and resulting protocol variations. We found the Compresstome® to be faster, allowing higher throughput and better use of the tissue samples available. We also found the use of the same agarose preparation used in protocol V1 performed better than the method suggested by the Precisionary Instruments protocol for these large sections of human lung.

Rationale

Diseases of the respiratory system are diverse in their onset, progression, and have an effect on all levels of complexity of an animal, from a subcellular level to the organism as a whole. Therefore, one could argue that a similarly diverse range of models should be available to researchers looking to study particular questions. One tool which has shown utility is Precision Cut Lung Slices (PCLS). PCLS have been used to investigate a variety of research questions in both human and animal studies. This includes mechanisms regulating airway contractility (1), and induction of pulmonary fibrosis (2), in addition to testing the toxicity of exposure to various chemical compounds (3).

At the University of Rochester Medical Center, we are in a unique position to prepare PCLS from donors in various states of disease progression and across a spectrum of ages and demographics. By providing sections which can be reliably thawed and placed into culture, researchers can begin to ask questions about how the tissue responds to exposure at different life stages or between groups with differing risk factors. In addition, PCLS have shown to be compatible with various -omic techniques making these a valuable tool for gathering a wide range of information about a particular sample.

Guidelines

Record details of procedure in Worksheet or Directly in BRINDL. The contents of the sample specific Worksheet is transferred to the BRINDL Database within the week. If documenting directly in BRINDL Database, please mark and file a worksheet with the Sample ID and statement of direct entry to track PCLS inventory.

Materials

Personal Protective Equipment (PPE):

1. Grossing Station / Fume Hood
2. Surgical Mask
3. Lab Coat
4. Nitrile Gloves
5. Face Shield
6. Biohazard Waste Bags
7. Sharps Container
8. OxyVir TB
9. Disinfecting Bleach

Other Equipment/Materials:

1. Double-edged stainless steel blades - Precisionary Instruments Part # VF-BL-VM-SSB
2. 50mL conical tubes or 120mL sterile specimen cups
3. Precisionary Instruments Compresstome® VF-510-0Z
4. Costar 24-well culture plates – Corning, Catalog # 3526
5. Dialysis tubing clamps (150mm) – Repligen, Catalog # 142253
6. Sterile cotton tipped applicators – Medline, Catalog # MDS202000
7. Sterile syringe (NO NEEDLE, at least 15 mL) –
8. Suture / small zip ties
9. Cyanoacrylate glue (super glue)
10. 10mm tissue coring tool - Alabama Research & Development, Catalog # MP0145
11. Gauze – Fisherbrand, Catalog # 22-246-069
12. Kimwipes – Kimberly-Clark, Catalog # 34155
13. Cryovials – Thermo, Catalog # 5000-1020
14. Stericup Vacuum Filter (150 mL) – Millipore, Catalog # SCGPU01RE
15. SureOne Filtered 1000µL Pipette Tips – Fisherbrand, Catalog # 02-707-404
16. LIVE/DEAD™ Cell Imaging Kit (488/570) - ThermoFisher, Catalog # R37601
17. CELLPRO-RO Cell Proliferation Reagent WST-1 - Millipore Sigma, Catalog # 05015944001

Chemicals:

1. Hanks' balanced salt solution (HBSS) – Corning; Catalog # 21-022-CV
2. Low-gelling point agarose – Sigma-Aldrich, Catalog # A9045
3. Dulbecco's Modified Eagle's Medium (DMEM), phenol-free – Gibco, Catalog # 21063-029
4. Antibiotic/Antimycotic – (Penicillin, Streptomycin, Actinomycin D) Corning, Catalog # 30004136
5. Fetal Bovine Serum (FBS, low endotoxin, preferably certified)
6. Dimethyl Sulfoxide – Sigma-Aldrich, Catalog # D8418-500ML



Safety warnings

! The tissue that is being used for preparation of PCLS is unfixed and therefore potentially infectious. Personal protective equipment (PPE) should be utilized to reduce the likelihood of exposure to any known or unknown pathogens within the sample. When cutting the PCLS, the vibratome blade is extremely sharp and should be handled with caution. Remove the blade holder when changing samples or replacing blades to reduce the risk of injury. DMSO, used in the freezing media, has the potential to increase the absorption of other chemicals into the body. Therefore, proper PPE should be used, particularly gloves and a lab coat. Change gloves immediately if contaminated. All institutional biosafety measures are followed in any manipulation of these human tissues.

Ethics statement

The protocols.io team notes that research involving animals and humans must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

Reagent Preparation

1 **2% (w/v) Agarose**

Cell culture suitable agarose (see Materials) is prepared in DMEM cell culture media for potential metabolic support of the agarose inflated tissue. The amount of agarose prepared will depend on the size of the lung lobe being inflated. Typical volumes are between 200 mL – 500 mL.

As an example, for a lung requiring 200 mL of 2% agarose, 4g of low gelling-point agarose is added to a sterile glass bottle and then enough DMEM is added to bring the volume up to 200 mL. This solution can be heated in a microwave in pulses, being careful not to boil over the beaker, until the agarose has melted into the DMEM. Place the bottle in a clean heated (37°C) water bath to keep the agarose **ABOVE 37°C** and **BELOW 40°C** to respectively prevent premature gelling of the agarose and cellular damage when instilled into the lung.

2 **Culture Media**

Prepare culture media by briefly thawing a 50 mL aliquot of FBS at 37°C. Filter this aliquot through a 0.22 µm vacuum filter into a new 500 mL bottle of DMEM. Finally, add 5 mL of the antibiotic/antimycotic reagent and mix the prepared media by closing the DMEM bottle and shaking. Advised to store the culture media at 4°C for **no more than 1 month** from the date of preparation.

3 **Freezing Media**

Freezing media is made up of 10% (v/v) of DMSO in FBS. The FBS should be filtered **BEFORE** adding DMSO, as the DMSO can cause problems with filtering.

4 **Warm Hank's Balanced Salt Solution**

In a clean histology water bath, heat to **37°C** a sufficient volume of HBSS to cover the agarose inflated lung. Do not warm to above **39-40°C**. This is done to prevent the agarose from hardening too quickly upon instillation into cold lung tissue.

Also prepare a container of ice cold HBSS to enhance agarose solidification in the lung after instillation.

Inflation of the Lung

- 5 Dissect the lobe of interest (typically one of the upper lobes) away from the en bloc lung as needed and cannulate its main bronchus with a large endotracheal tube, or similar catheter, to fit the size of the main bronchus. Secure the tube in place with suture or zip-tie to prevent dislodgement and leakage. The tube should be inserted as deeply as possible, but do not bypass the first airway branch point to ensure uniform inflation of the lobe. For larger adult lobes, a dialysis clamp may be used to reduce the extent of the lobe that will be agarose inflated.

- 6 Place the cannulated lobe into the warmed (**37°C**) HBSS for 5 minutes to bring the lobe up to temperature prior to inflation to avoid the agarose gelling upon entry into the lung.
- 7 When 5 minutes have elapsed, keeping the lobe in the warm HBSS, fill your syringe with the prepared 2% agarose and connect this to the end of the endotracheal tube. Slowly apply pressure to begin inflation, refilling the syringe with warm agarose as needed. Being sure to keep 2% agarose solution at 37°C, while trying to move quickly and gently. While moderate firmness of the inflated lung is desired, take care not to over inflate the tissue as the alveolar septae can be significantly stretched and thinned with over inflation.
- 8 When the lobe (or lobe part) has become moderately firm by inflation, the cannulating tube is removed as the suture / zip-tie is tightened to secure the airway and prevent leakage and deflation. If a clamp was used to reduce amount of tissue instilled with agarose, leave this in place.
- 9 Place the lobe into ice-cold HBSS and keep any floating portions of the lobe moist with gauze for at least 45 minutes, until the lobe solidifies.

Slicing of the Agarose Inflated Lung

- 10 Remove the lobe from the ice-cold HBSS and slice the lobe, approximately 0.5-1cm thick, according to already established protocol (dx.doi.org/10.17504/protocols.io.biz7kf9n).
- 11 When selecting slices for culture, make sure that the slices are well inflated with agarose.
- 12 Place the selected slices into either 50 mL sterile conical tubes or 120 mL sterile specimen cups, depending on the size of the slice, containing enough Culture Media to cover the tissue fully.
- 13 Store overnight at 4°C until sectioning. This step has been shown to improve subsequent viability.

Coring and Sectioning of the Lung Slice

- 14 Place chilling block for Compressstome® specimen tube into freezer to cool for at least 1 hour prior to cutting tissue.
- 15 Cool HBSS to 4°C by placing it in the refrigerator the night before or in the freezer for about 15 minutes while setting up the Compressstome® (avoid freezing the HBSS).

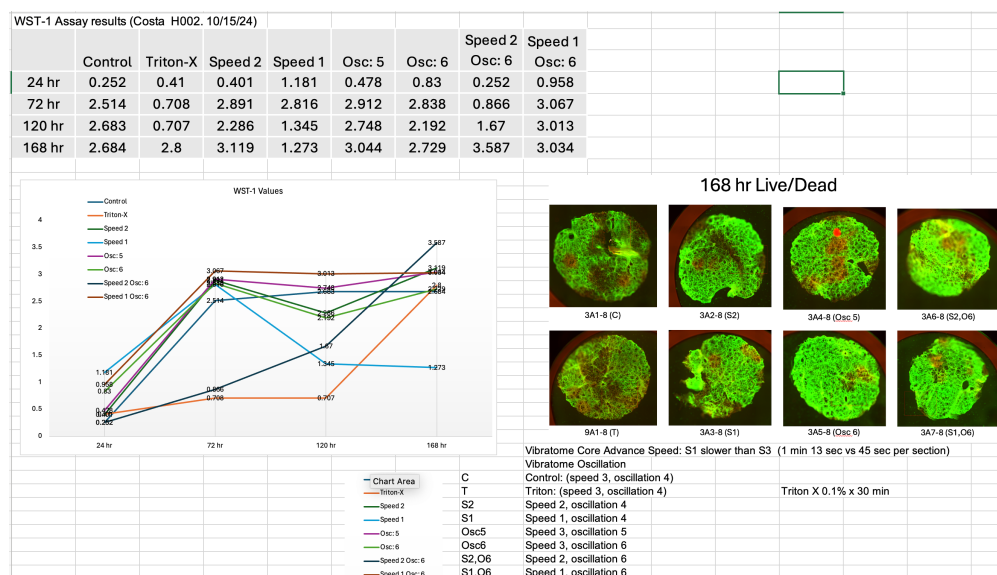
- 16 Prepare 25ml of 2% agarose in a sterile glass bottle, and keep bottle in a preheated (37°C) water bath to prevent gelling of agarose.
- 17 Set up the Compresstome® in a room with minimal interruptions. For safety it is suggested that anyone entering the room wears at minimum a lab coat, gloves, face shield and an surgical mask due to the risk of aerosol generation from the sectioning.
- 18 Keep the agarose inflated lung slices cool (in media on wet ice) throughout the process of creating PCLS.
- 19 Remove a slice from its respective container and carefully use a 10mm tissue coring tool to create a core of lung tissue, and place the remainder of the slice back into the cold culture media until the next core is made so it does not dry out.
- 20 Carefully blot the tissue core dry with a kimwipe. Add a drop of cyanoacrylate glue to the center of the Compresstome® specimen tube base. Using forceps, place the tissue core on the adhesive, holding it there until the glue is cured (about 20 seconds)
- 21 Pull the specimen tube base downwards until the core fully enters the metal tube, leaving a small amount of space above the core.
- 22 Using a disposable pipette, carefully add melted agarose (**37°C**) to the tube to fully cover the core. Hold the metal tube tightly so the base does not move while the agarose cools.
- 23 Take the cold chilling block and place it over the specimen tube for about 1 minute to help the agarose solidify. Put the chilling block back into the freezer (or a bucket of ice) to stay cold until the next sample needs to be made.
- 24 Once the agarose around the core is solidified, push the sliding control box of the Compresstome® back away from the buffer tray and insert the specimen tube into the buffer tray.
- 25 Bring the sliding control box forward, and move the micrometer forward using the "fast forward" function until it is touching the back of the specimen tube. It is ok if the micrometer slightly pushes the specimen tube base forward, this ensures that it is fully touching the base.
- 26 Fill the buffer tray with enough cold HBSS that it covers the specimen tube. In between cutting new cores, the HBSS should be drained and replaced with fresh cold HBSS if it has warmed up.
- 27 Slide a prepared blade holder onto the compresstome with the side holding the blade facing the control box/specimen tube and tighten into place using the Allen key provided with the unit.

Assessment of Viability

28 Precisionary Instruments recommended starting settings when sectioning:

- i. Advance - 2 to 3
- ii. Oscillation - 3 to 4
- iii. Beginning Thickness - 550µm
- iv. Final thickness of saved sections - 500µm

On human agarose inflated lung, our lab trialed oscillations 4 to 6 and advance (speed) 1 to 3 followed by testing of viability in culture by WST-1 Assay and Calcein AM/Ethidium Homodimer (Live/Dead) staining after culture for 24 to 168 hours. Results showed slightly improved viability when using Advance (Speed) 1 with Oscillation 6 as compared to the manufacturer's suggested settings.



Example WST-1 and CalceinAM-EthidiumHD Live-Dead Assays at time points after freeze-thaw of PCLS

Assessment of Viability

- 29 To produce slices with consistent thickness, start the sectioning thickness at 550µm. After each slice is produced, reduce the thickness by 10µm until a full face of the core is cut, or until 500µm is reached. All sections made before the 500µm slice should be discarded due to irregularity.
- 30 Use a new sterile cotton tipped applicator to transfer sections from the specimen well into a HBSS filled 24 well tissue culture plate kept chilled on ice.

Preservation of PCLS

1d

- 31 After a culture plate is full, the sections are placed into a fresh plate, prewarmed in a 37°C incubator, with 1 mL of Culture Media in each well. Incubate overnight.
- 32 The next day, these sections can be frozen down by placing them in labeled cryovials with 1 mL Freezing Media per section. These vials will go into a -80°C freezer using a Mr. Frosty to control the rate of freezing.
- 33 After 24 hours in the -80°C freezer, these sections are transferred to a liquid nitrogen freezer.

1d

PCLS Thaw and Culture

- 34 Remove PCLS vial from liqN2 freezer. Thaw by holding in fingers or by brief period in 37deg water bath - keep this thaw time to minimum.
- 35 Place the PCLS section in 5 ml of Culture Media (DMEM+10%FBS+1% Antibiotic/mycotic). Wash x 3 in Culture Media then place in fresh media in fresh culture dish before placing in incubator.
- 36 Recommend at least 24 hour resting culture in 37°C incubator prior to beginning experiments based on time needed for recovery of WST-1 mitochondrial dehydrogenase activity (see above).

Assessment of Viability

- 37 The WST-1 Assay is a colorimetric assay used to asses the viability of cells. It is dependent on live-cell mitochondrial dehydrogenase activity to form formazan dye from tetrazolium salt. The amount of formazan detected by spectrophotometry is directly expected to be proportional to cell viability.
The Calcein AM/Ethidium Homodimer stain is a Live/Dead stain used to differentiate between live and dead cells in a population using fluorescent dyes. Calcein AM stains live cells green following conversion by intracellular esterase acitivity while the Ethidium Homodimer can only enter dead cells, staining their DNA red. These assays were performed according to the supplier's protocols, for quality assessment of viability.
- 38 The viability of these sections has been analyzed using a variety of techniques including the WST-1 assay and Live/Dead fluorescence imaging (See example above). Good to excellent viability appears consistent at least up to one week, extended viability time course assays should be tested as needed for experimental design.

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