Journal of Visualized Experiments

Handling of the cotton rat in studies for the pre-clinical evaluation of oncolytic viruses

--Manuscript Draft--

Manuscript Number:	JoVE52232R2
Full Title:	Handling of the cotton rat in studies for the pre-clinical evaluation of oncolytic viruses
Article Type:	Methods Article - JoVE Produced Video
Keywords:	cotton rat; oncolytic virus; animal handling; bovine herpesvirus type 1
Manuscript Classifications:	2.4: Viruses; 3.4: Neoplasms; 5.2: Therapeutics
Corresponding Author:	Karen Mossman McMaster University Hamilton, Ontario CANADA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	mossk@mcmaster.ca
Corresponding Author's Institution:	McMaster University
Corresponding Author's Secondary Institution:	
First Author:	Breanne Cuddington
First Author Secondary Information:	
Other Authors:	Breanne Cuddington
	Meghan Verschoor, PhD
Order of Authors Secondary Information:	
Abstract:	Oncolytic viruses are a novel anticancer therapy with the ability to target tumor cells, while leaving healthy cells intact. For this strategy to be successful, recent studies have shown that involvement of the host immune system is essential. Therefore, oncolytic virotherapy should be evaluated within the context of an immunocompetent model. Furthermore, the study of antitumor therapies in tolerized animal models may better recapitulate results seen in clinical trials. Cotton rats, commonly used to study respiratory viruses, are an attractive model to study oncolytic virotherapy as syngeneic models of mammary carcinoma and osteosarcoma are well established. However, there is a lack of published information on the proper handling procedure for these highly excitable rodents. The handling and capture approach outlined minimizes animal stress to facilitate experimentation. This technique hinges upon the ability of the researcher to keep calm during handling and perform procedures in a timely fashion. Finally, we describe how to prepare cotton rat mammary tumor cells for consistent subcutaneous tumor formation, and how to perform intratumoral and intraperitoneal injections. These methods can be applied to a wide range of studies furthering the development of the cotton rat as a relevant pre-clinical model to study antitumor therapy.
Author Comments:	We will need a few weeks notice prior to filming, if accepted, as we need to order in cotton rats, as we do not breed these animals in house. Thank you.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to	

author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

Title:
Handling of the cotton rat in studies for the pre-clinical evaluation of oncolytic viruses

Authors:
Breanne Cuddington, Dr. Meghan Verschoor and Dr. Karen Mossman

Authors: institution(s) and affiliation(s) for each author:
Breanne Cuddington
Department of Pathology and Molecular Medicine,
McMaster Immunology Research Centre,
Institute for Infectious Disease Research
McMaster University
cuddingb@gmail.com

Dr. Meghan Verschoor
Department of Pathology and Molecular Medicine,
McMaster Immunology Research Centre,
Institute for Infectious Disease Research
McMaster University
meghan.verschoor@gmail.com

Dr. Karen Mossman
Department of Pathology and Molecular Medicine,
McMaster Immunology Research Centre,
Institute for Infectious Disease Research
McMaster University
mossk@mcmaster.ca

Corresponding author:
Dr. Karen Mossman, mossk@mcmaster.ca

Keywords: cotton rat, oncolytic virus, animal handling, bovine herpesvirus type 1

Short abstract:
Cotton rats are extremely excitable and have a strong flight-or-fight response. A handling method optimized to reduce the stress of the animals is described which will make cotton rats more accessible as a preclinical model.

Long abstract:
Oncolytic viruses are a novel anticancer therapy with the ability to target tumor cells, while leaving healthy cells intact. For this strategy to be successful, recent studies have shown that involvement of the host immune system is essential. Therefore, oncolytic virotherapy should be evaluated within the context of an immunocompetent model. Furthermore, the study of antitumor therapies in tolerized animal models may better recapitulate results seen in clinical trials. Cotton rats, commonly used to study respiratory viruses, are an attractive model to study oncolytic virotherapy as syngeneic models of mammary carcinoma and osteosarcoma are well established.

However, there is a lack of published information on the proper handling procedure for these highly excitable rodents. The handling and capture approach outlined minimizes animal stress to facilitate experimentation. This technique hinges upon the ability of the researcher to keep calm during handling and perform procedures in a timely fashion. Finally, we describe how to prepare cotton rat mammary tumor cells for consistent subcutaneous tumor formation, and how to perform intratumoral and intraperitoneal injections. These methods can be applied to a wide range of studies furthering the development of the cotton rat as a relevant pre-clinical model to study antitumor therapy.

Introduction:

Oncolytic viruses (OV) selectively replicate in tumor cells by exploiting biochemical differences between normal and tumour cells [1,2]. There are two types of OVs: those that do not require a mutation to achieve selective oncolysis, referred to as naturally occurring wild-type viruses and those which must be engineered to achieve selective oncolysis. The collection of mutations within a given tumor type determines the nature of the selective growth advantage over normal cells for an OV [2]. The safety and benefit of using OVs has been demonstrated in clinical trials [3-7]. Despite advancements in the field of oncolytic virotherapy there exist gaps between pre-clinical and clinical results, suggesting that better models are needed to evaluate the antitumor efficacy of OVs.

Bovine herpesvirus type 1 (BHV-1) is a member of the *Herpesviridae* family, and *Alphaherpesviridae* subfamily. BHV-1 initiates bovine respiratory disease complex in cattle, manifesting in a wide variety of symptoms resembling a bad cold [8,9]. BHV-1 binds attachment and entry receptors used by HSV-1, such as heparan sulfate and nectin-1 [10]. However, it binds CD155 in the place of nectin-2 [10]. BHV-1 has a very narrow host range such that it is unable to efficiently enter and initiate replication in normal and transformed murine cells [3,4,10]. This makes the use of conventional murine models problematic. The oncolytic capacity of BHV-1 has been demonstrated *in vitro* [11,12]. BHV-1 has been shown to initiate replication in and kill human tumor cells from a variety of histological origins, including breast cancer cells and breast cancer initiating cells [11,12]. However, the antitumor capacity of BHV-1 must be evaluated *in vivo* within the context of an immunocompetent host.

Human Adenovirus (Ad), for which there are 57 identified serotypes, most commonly causes respiratory illness in humans. Oncolytic Ad vectors have been evaluated for their antitumor efficacy with several advancing into clinical trials [13-15]. Despite promising pre-clinical data, clinical results have fallen short of expectations. Human tumor xenograft models are typically used to study the antitumor efficacy of Ad vectors, although they exhibit attenuated immune responses to the virus [16,17]. Furthermore, syngeneic murine models are non-permissive to Ad infection, making the evaluation of host immune responses using these models impractical [17,18].

The host immune system has been identified as the most influential mechanism by which OVs elicit tumor cell death [19]. Antitumor responses between tolerized and non-tolerized tumor-associated antigen (TAA) models differ and can greatly impact the success of OV therapy. The HSV-1 OV KM100 (ICP0^{n212}VP16$^{in1814\ 20}$)[20,21] elicited tumor regression in 80% of tumor-bearing mice in a murine Polyoma Middle T antigen mammary cancer model [22]. However, in HER-2/neu models, the antitumor efficacy of KM100 varied between 20% complete regression

in syngeneic mice and tumor stasis in transgenic, HER2-tolerized mice. Together these data highlight the importance of fully evaluating OVs using animal models that best recapitulate the human immune landscape to fully understand what features determine therapeutic success.

The cotton rat (*Sigmodon hispidus*), indigenous to North and South America, is most commonly used as a model of respiratory syncytial virus infection (as reviewed in [5]). Cotton rats are also used in anti-BHV-1 vaccination research as they recapitulate the pathology associated with bovine respiratory disease complex [6,23]. Furthermore, BHV-1 infection of cotton rats is immunogenic, inducing sustained mucosal and systemic immune responses [6,23-25]. Cell lines have been derived from spontaneous fibrosarcoma and osteosarcomas of the mammary gland (LCRT) and bone (CCRT and VCRT), respectively [26]. Cotton rats have been used to evaluate the *in vivo* efficacy of oncolytic Ad vectors as they are susceptible to Ad infection and exhibit similar pathology to humans [27-29]. The use of immunocompromised models for the pre-clinical evaluation of OVs are not only less indicative of clinical responses to therapy but they fail to take into account the role of the immune system in oncolytic virotherapy [30,31]. Therefore, the syngeneic and tumour-tolerized cotton rat models of mammary carcinoma and osteosarcoma are relevant models in which to evaluate the pre-clinical efficacy of OVs, such as BHV-1 and Ad which cannot be studied using conventional murine models.

Protocol Text:
The protocols used have been approved by our institutional Animal Research Ethics Board at McMaster University according to Canadian Council on Animal Care guidelines. Experiments were performed at the McMaster University Central Animal Facility.

1. Culturing LCRT Cells

1.1) Culture LCRT cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Maintain cells in T-150cm^2 tissue culture flasks at 37 ^{o}C and 5% CO_2. Passage cells when they form a 90% confluent monolayer (every 2-3 days, **Figure 1**).

1.2) Pre-warm 1X phosphate buffered saline (PBS), 1X trypsin and medium in a 37 ^{o}C water bath for 10 min prior to splitting the cells.

1.3) Aspirate medium from the flask and rinse cells with 5 mL of 1X PBS.

1.4) After rinsing, aspirate PBS and incubate cells with 2 mL of 1X trypsin until cells dissociate from the flask (~2 min).

1.5) Resuspend cells in 8 mL medium (for a total of 10 mL cell suspension) and gently pipette up and down to break up clumps of cells.

1.6) Maintain cells in a T-150 flask by seeding 1 mL cell suspension into 24 mL medium (for a total of 25 mL per T-150) and rock flask gently from side to side. Maintain cells at 37 ^{o}C and 5% CO_2 until next split.

2. Assessing virus replication and cytotoxicity in LCRT cells

2.1) Virus Replication

Virus constructs expressing a fluorescent tag, such as green fluorescent protein (GFP), under control of endogenous viral promoters facilitate visualization of virus infection and spread using fluorescence plate readers.

2.1.1) Seed LCRT cells into culture plates, leaving a well for counting. Seed cells such that they will be 80-90% confluent one day later. Use a concentration of 10^5 cells/mL (100 µL per well) to produce the desired confluency one day later in 96-well flat bottom plates.

2.1.2) The next day, pre-warm 1X PBS, 1X trypsin, complete and serum-free medium in a 37 ^oC water bath for 10 min prior to starting the experiment.

2.1.3) Aspirate medium from the counting well and rinse cells with 5 mL of 1X PBS by rocking it over the surface of the well.

2.1.4) After rinsing, aspirate PBS and incubate cells with 2 mL of 1X trypsin until cells dissociate from the flask (~2 min).

2.1.5) Resuspend the cells in the appropriate volume of complete medium to yield a cell density within the countable range using a hemocytometer. To ensure an accurate cell count, thoroughly mix the cell suspension prior to inoculating the hemocytometer by pipetting up and down.

2.1.6) Determine the volume of virus stock required for infection at the desired multiplicity of infection (MOI).
Required Paque Forming Units (pfu) = number of plated cells * MOI (pfu/cell)
Volume of virus stock required = required pfu / virus stock titre (pfu/mL)

2.1.7) Prepare virus inoculum in serum-free medium in tubes. Mix thoroughly by vortexing or pipetting before adding inoculum to the cells.

2.1.8) Infect cells for 1 hour at 37 °C, after which apply a maintenance overlay of DMEM+1% FBS.

2.1.9) Scan plates one and two days post infection (pi) to visualize GFP fluorescence.

2.2) Virus Cytotoxicity

NOTE: Perform the resazurin cytotoxicity assay under low light conditions as the compound is photosensitive. A well containing medium only should be included to correct for background fluorescence.

2.2.1) Prepare a 5% (v/v) solution of resazurin in 1X PBS. Mix the solution by pipetting.

2.2.2) Aspirate medium from cells and apply the 5% resazurin solution. Include a well containing medium only to correct for background fluorescence.

2.2.3) Incubate cells for 30 min at 37^{o}C, after which read fluorescence using a fluorescence plate reader (excitation 530 nm, emission 595 nm).

2.2.4) Analyze data relative to uninfected controls correcting for background fluorescence.

3. Housing and Handling

3.1) Housing and Diet

3.1.1) House cotton rats individually to decrease in-fighting in polycarbonate rat cages containing rodent bedding (1/8" corncob bedding), a section of PVC tubing no longer than 8 inches and nestlets as enrichment (**Figure 2)**.

3.1.2) Use a securable steel basket to sit overtop of the cage and contain rodent food and a water bottle.

NOTE: This cage setup will allow for safe and easy capture of the animals, with the placement of the enrichment tube against the end of the cage being of the utmost importance.

3.2) Handling

3.2.1) Handle cotton rats in the morning, prior to rounds by animal facility technicians to avoid exciting them before a procedure.

3.2.2) During all procedures, wear thick leather gloves for protection.

3.2.3) As the animals primarily remain in the enrichment tubes, use them to transfer the rats into a new cage during routine cleaning. Alternatively, open the cage slightly to allow the handler to reach their hand in, the animal can then be restrained by scruffing the skin just above the shoulders and pushing down. Practice care not to use excessive force as the animal may bite their tongue.

3.2.4) Be patient and use a steady hand as the animals have a strong flight-or-fight response and will try to avoid capture by running and jumping out of the cage. Importantly, do not handle animals by the tail as degloving will occur.

3.2.5) Trap the animals in their enrichments tubes over direct handling. This drastically decreases injuries and escaping.

4. Capture and Anesthesia

4.1) Capture

4.1.1) Wear thick leather gloves for protection during all procedures.

4.1.2) Use a large clear plastic container with holes for air and a lid, an anesthetic induction chamber large enough to fit the container and a nose cone fitted to the gas output hose (**Figure 3**).

4.1.3) Work in pairs to make the procedure more efficient and to decrease the exposure time of the animals to isoflurane, an inhalation anesthetic. Make sure one researcher is responsible for opening and replacing the steel cover on the cage and the lid of the induction chamber (handler #1) and their associate is responsible for capture of the animal in the tube and transport to the induction chamber (handler #2).

4.1.4) Place the cage on a flat surface and remove the outer lid. Lift the steel feed tray slightly and slowly manoeuvre the enrichment tube so it is parallel with the sides of the cage and against the back. If necessary, use an object to manoeuvre the enrichment tube without opening the cage to avoid agitating the animal (handler #1)..

4.1.5) If the animal becomes agitated and leaves the tube, allow adequate time for the animal to relax and once again settle in the tube (handler #1)..

4.1.6) Slowly and deliberately lift the edge of the steel cover furthest from the enrichment tube, keeping the other end in contact with the cage. Make a space large enough for the plastic container (handler #2).

4.1.7) In one smooth, quick motion, push the plastic container overtop of the enrichment tube. Maintain contact of the container with the side of the cage, trapping the animal in the tube. Perform steps 4.1.6 and 4.1.7 as quickly as possible (handler #2).

4.1.8) Remove the steel feed tray and give the plastic container lid to handler #2 (handler #1). Slide the plastic lid between the side of the cage and the container, being mindful of the trapped animal's appendages in the process. Do not seal the container as this will make the next step more difficult (handler #2).

4.2) Anesthesia

4.2.1) Make sure the container remains closed and transport the animal to the induction chamber. Quickly place the animal in the chamber and remove the container lid in one fluid motion (handler #2). Open and immediately replace the induction chamber lid (handler #1).

4.2.2) Turn on the flow of isoflurane to the induction chamber (5 L/min) and monitor the animal for signs of lethargy, at which point quickly slide the animal from the tube and container, removing both from the induction chamber to facilitate gas circulation.

4.2.3) When the rat is fully anesthetized, move it to the working surface and place the nose and mouth into the nose cone (**Figure 3**). The rat is fully anesthetized when it is unresponsive to a forceful toe pinch.

4.2.4) Place vet petrolatum ophthalmic ointment on the animal's eyes to prevent dryness and abrasions. This is an essential step as cotton rats have large eyes that can be prone to infection if injury occurs.

4.2.5) Carefully monitor and maintain a constant respiration rate and ensure that the animal's nose remains in the nose cone fitting throughout the procedure. Adjust the flow rate of isoflurane appropriately. The amount of isoflurane required to anesthetize each animal will vary.

4.2.6) Post-procedure, return the animal to its cage and ensure it regains full mobility and sternal recumbency.

5. Preparation of LCRT cells for subcutaneous tumor formation

One T-150 flask of LCRT (90% confluency) yields approximately $2x10^7$ cells. Base the number of T-150 flasks required on the total number of cells needed. Seed extra flasks to ensure the total number of cells required is obtained and to accommodate cells lost during preparation and those needed for extra injections. Keep cells on ice whenever possible to prolong cell viability.

5.1) To harvest cells, aspirate medium from the flask and rinse cells with 5 mL of 1X PBS.

5.2) Aspirate PBS and incubate cells with 2 mL of 1X trypsin until cells dissociate from the flask (~2 min).

5.3) Resuspend cells in 8 mL medium (for a total of 10 mL cell suspension) and gently pipette up and down to break up clumps of cells. Continue to harvest cells from additional flasks.

5.4) Pool all cell suspensions into one conical tube, approximately 4 T-150s per 50 mL conical tube.

5.5) Centrifuge the tube at 160 x *g* for 10 min at 4 ºC.

5.6) Aspirate medium and resuspend the cell pellet in the appropriate volume of PBS (10 mL PBS per T-150) to yield a cell density within the countable range using a hemocytometer. To ensure an accurate cell count, thoroughly mix the cell suspension prior to loading the hemocytometer by pipetting up and down.

5.7) Calculate the total number of cells:
Total number of cells harvested = cell count (cells/mL) x resuspension volume (mL)

5.8) Determine the volume of cell suspension required for all injections. Make 2-3 extra doses per experiment. A total of 5×10^5 LCRT cells injected subcutaneously will form palpable tumors within 3-4 days.

Total number of cells required = $5x10^5$ cells x total number of doses
Volume cell suspension required (mL) = (total cells required * sum of injection volumes) / (total number of cells harvested)

5.9) Pipet the required volume of cell suspension into a conical tube containing PBS and mix thoroughly. Aliquot individual injections (100 µL) into eppindorf tubes. Maintain tubes on ice during injection procedure.

6. Injections

Perform procedures with two researchers, one to perform the injections while the other monitors the animal's respiration rate and general condition while under anesthesia. Use insulin syringes (29 G x 1/2', 0.3 mL) for all injections and a new needle for each animal.

6.1 Subcutaneous injections

6.1.1) Capture and anesthetize the animal (section 4).

6.1.2) Shave the injection site using clippers. Cotton rat fur is thick and requires a sharp trimmer to get a smooth surface for injections. Clean the injection site with 70% ethanol using a cotton swab and allow it to evaporate completely before proceeding.

6.1.3) Load syringes (29 G x 1/2', 0.3 mL) with the cells by drawing up slowly and steadily. If bubbles are evident flick the syringe with some force. Once the bubbles are at the top push the plunger until the liquid is at the top of the needle.

6.1.4) Lift the skin at the injection site (referred to as skin tenting) and insert the needle bevel side up. Make sure the needle moves freely under the skin to avoid injecting intramuscularly.

6.1.5) Expel the contents of the syringe evenly and slowly. Withdraw the needle bevel side down.

6.2 Intratumoral injections

6.2.1) Capture and anesthetize the animal (section 4).

6.2.2) Clean the injection site with 70% ethanol using a cotton swab and allow it to evaporate completely before proceeding.

6.2.3) Load syringes (29 G x 1/2', 0.3 mL) with the virus inoculum by drawing up slowly and steadily while holding the needle in an upright position. If bubbles are evident flick the syringe with some force. Once the bubbles are at the top push then plunger until the liquid is at the top of the needle.

6.2.4) Insert the needle bevel side up into the tumor and expel the contents of the syringe evenly and slowly while moving the needle in a fan-like pattern, partially withdrawing the needle before each movement to prevent laceration of the tumor. Withdraw the needle bevel side down.

NOTE: Subcutaneous LCRT tumors are fast-growing, reaching approximately 100 mm^3 in 5-7 days. Furthermore, necrotic and hemorrhagic centers often form on the surface of the tumor within several days and require careful monitoring (**Figure 4)**.

6.3 Intraperitoneal injections

6.3.1) Capture and anesthetize the animal (section 4).

6.3.2) Clean the injection site with 70% ethanol using cotton swabs and allow it to evaporate completely before proceeding.

6.3.3) Load syringes (29 G x 1/2', 0.3 mL) with the drug by drawing up slowly and steadily. If bubbles are evident flick the syringe with some force. Once the bubbles are at the top push then plunger until the liquid is at the top of the needle.

6.3.4) Insert the needle into the right lower quadrant of the abdomen. Pull back on the plunger to ensure that blood or feces are not aspirated, this indicates incorrect placement of the needle. If this occurs, withdraw the needle and prepare a new syringe. When the needle is correctly placed, expel contents of the syringe evenly and slowly.

7. Tumor excision and necropsy

7.1) Gather and clean all tools with 70% ethanol prior to euthanasia of the animal.

7.2) Euthanize the animal by the desired method, CO_2 inhalation (2 L/min for 5-10 min) is recommended. Examine the animal for any abnormalities in body condition.

7.3) Place the animal in dorsal recumbency on a dissection board and clean the animal with 70% ethanol.

7.4) Use tweezers to lift the skin at the lower abdomen. Cut through the skin and muscle using scissors and make a medial incision running the length of the animal (anus to chin).

7.5) Cut the rib cage by making two cuts, one laterally up the side of the ribcage and one across the sternum to expose the heart and lungs. Examine the lobes of the lung for any metastases [27,29].

7.6) Examine all organs for abnormalities and record any changes in color, size, and consistency. If necessary, incise the organs with a scalpel to examine internal tissues. Specifically, examine the liver, kidneys, spleen and gastrointestinal tract.

7.7) Inspect the lymph nodes for metastases and enlargement [27,29].

7.8) To collect the tumor, make flank incisions above and below the tumor such that the skin can be pulled away from the body with tweezers. While firmly holding the skin with tweezers, use a scalpel to carefully remove the tumor by cutting between the tumor and dermis (**Figure 5)**.

7.9) Immediately place the tumor in a labelled container of 10% neutral buffered formalin.

7.10) Depending on the size of the tumor, allow 1-2 days (≤ 2 mm, small) or 5-6 days (> 2 mm, large) for fixing before preparing sections for histological analysis (**Figure 6**).

Representative Results:
Due to the extremely excitable nature of cotton rats, being familiar with and utilizing procedures optimized to reduce the stress of the animals will ease in their use as a pre-clinical animal model. Use of proper handling techniques will also minimize risk to the researcher.

When using cotton rats it is imperative to stay calm. The rats are highly excitable and will attempt to escape their cage. Use of an enrichment tube and nestlets will minimize escape attempts. **Figure 2** shows optimal cage setup to aid in capture of cotton rats, including placement of the enrichment tube. Furthermore, work in a small room if possible to aid in recapture. If escape occurs, wait for the animal to calm down and remain stationary, then cover it with the clear capture container or cover with gloved hands, being careful not to use excessive force.

In contrast to a mouse, the cotton rat has an elongated snout which requires a different nose fitting to deliver the anesthetic gas. **Figure 3** depicts a nose cone engineered to properly fit a cotton rat and maximize delivery of isoflurane. Using a rubber membrane as a fitting may result in trauma to the nose of the rat.

If possible obtain discard animals (those not needed by other researchers, cotton rats or otherwise) to practise injection techniques prior to attempting them on the rats. This will allow the researcher to gain familiarity with needles and how to safely handle them. Insulin syringes are suggested for injections in cotton rats as their skin is thick and tough in comparison to a mouse. However, a larger needle (21 G x 1')can be used for the injection of tumor cells to avoid loss of cell viability due to cell shearing during injection. Safety precautions should be followed, such as not recapping needles and proper disposal into a sharps container.

The injection of viable tumor cells is important for proper tumor formation. **Figure 1A** shows a healthy monolayer of LCRT cells which can be prepared for injection into cotton rats. In comparison **Figure 1B** shows LCRT cells which have low viability and should not be used for injections. It is important to verify tumor cell viability using a method such as trypan blue staining when counting cells for injections.

The tumors formed from LCRT cells are fast-growing and necrotic centers often form (**Figure 4A**). As such, tumor formation should be monitored carefully to avoid ulceration (**Figure 4B**). If ulceration occurs the animal should be sacrificed to avoid infection and possible death from sepsis.

The effects of anti-tumor treatments are often best examined through histological analysis. This requires excision of the tumor post mortem. Maintaining tumor tissue integrity will result in a sample which is a more accurate representation of the tumor *in vivo*. **Figure 5** shows an excision technique by which the tumor is carefully separated from surrounding tissue using a scalpel and tweezers. Removing the tumor by pulling it by force from the surrounding tissue using tweezers

may rupture the tumor or disrupt tumor tissue integrity, impacting proper histological analysis. The dense and highly vascularized structure of the tumor, as seen in **Figure 6**, is maintained by this excision technique. This is important in analysis of treatments which affect tumor vasculature, as is the case with many OVs.

Figure Legends:

Figure 1: Bright-field microscopy image of LCRT Cells. A) Phenotype of healthy (~90% viable) LCRT cells ready for preparation for injection into cotton rats. B) Undesirable phenotype of LCRT cells not fit for injection. Rounded cells are dead or dying (indicated by an arrow). Images were captured at 10x magnification; scale bar = 1 mm.

Figure 2: Example of cage setup for ease of capture of cotton rats. Optimal placement of enrichment tube against the end of the cage and inclusion of nestlets aids in animal capture.

Figure 3: Anesthesia nose cone fitting for delivery of isoflurane to anesthetized cotton rats. Manufactured nose cone fits elongated snout of cotton rat to ensure accurate delivery of isoflurane gas without trauma to nose.

Figure 4: Necrotic tissue on a subcutaneous LCRT tumor. A) Early stages of necrosis of tumor tissue. The animal should be carefully monitored to avoid progression to B) fully open ulceration. The animal should be sacrificed if tumor ulcerates as infection and sepsis can result.

Figure 5: Excision of a subcutaneous LCRT tumor for histology. The subcutaneous tumor on the flank of a cotton rat is carefully removed from the skin using a scalpel to maintain tumor tissue integrity, thus providing a better representation of tumor architecture for histological analysis.

Figure 6: Histological tissue section from a subcutaneous LCRT tumor. The morphology of LCRT tumor tissue examined using paraffin-embedded sections stained with hematoxylin and eosin (H&E). Image was captured at 20x magnification; scale bar = 1 mm.

Discussion:

Cotton rats are highly excitable and have a strong flight response. Therefore, special care should be taken to minimize any undue stress on the animal. The cage setup described will allow for safe and easy capture of the animals, with the placement of the enrichment tube being of the utmost importance. When setting up cages, ensure that the enrichment tubes meet the size and shape requirements, and are placed in proper orientation in the cage. It is also important to ensure that any technicians who might be aiding in animal maintenance are informed of the specific requirements for housing. Taking these precautions will reduce agitation of the animal prior to performing procedures. It is also advisable that procedures be performed in the morning, before any handling by technicians or animal facility staff. Commencing procedures with calm animals will aid in their capture and handling.

The use of nestlets is highly recommended. In the wild, these rodents make their nests out of cotton so they are more comfortable when given the option to build them in their cages. A nest can also aid in animal capture, such that the presence of the animal in the enrichment tube may no longer be required. In this case, the plastic container may be placed directly on top of the

animal from above. This action is of particular use as an experiment progresses as the rats may adapt to avoid the traditional capture method.

When handling cotton rats it is of the utmost importance that the researcher remain calm and make deliberate movements. These animals can sense unease and will respond to the perceived emotional state of the handler. Wearing thick leather gloves, following the procedures as described and approaching them calmly and with confidence will protect from bite injuries.

There are conflicting reports in the literature regarding the response of cotton rats to anesthetics [26,32]. Sedation of cotton rats for extended periods of time using isoflurane is not recommended [32]. We have found that, with careful monitoring, animals can be sedated using isoflurane for approximately 15-20 minutes without adverse effects. In our experience the rats respond rapidly to isoflurane and are fully sedated within several minutes. Likewise, waking occurs in less than a minute in most cases. However, anesthetizing an animal under intense stress alters their response to isoflurane, such that during waking they experience a lack of hind limb motor control and will stumble around the cage. This effect does not appear to have any lasting impact as the animal can execute proper motor control within several minutes of recovery.

When preparing the LCRT cells for tumor injections, it is imperative that the cells be kept on ice whenever possible to prolong viability. Mix the cells with a large bore pipet to avoid shearing the cells. Furthermore, to ensure that all tumors grow at a similar rate we recommend staggering the preparation of multiple cell suspensions when more than five animals are being injected. This will avoid slower growth of tumors in animals injected last due to decreased viability of the LCRT cells.

Excising the tumor tissue from the skin using a scalpel better maintains the tumor architecture and vasculature for histology in comparison to using tweezers to pinch the tumor from the skin. Although perfusion with phenobarbital will best preserve tissues for histopathology, obtaining a license for barbiturates was a limiting factor and was not performed. Thus, necropsies were performed immediately following euthanasia to preserve tissues. In our experience, this allowed for an accurate assessment of the pathology associated with our treatment.

Overall, the major limiting factor is the requirement for two researchers for handling procedures. Although the procedures can be performed by one handler, the process is greatly facilitated, and the stress of the animals significantly reduced, using two handlers. These procedures require practice and careful planning. Allow sufficient time to carry out methods calmly and deliberately. There exists significant stigma about the aggressive nature of these animals and the difficulty in their handling. This article serves to make these animals more accessible to researchers by detailing a simple, established handling method that effectively minimizes stress and injury to both the researcher and the animals. Using the methods we have described, cotton rats represent an excellent animal model for the study of many immune therapies, including oncolytic virotherapy.

Acknowledgements:
Breanne Cuddington holds a fellowship from the Canadian Breast Cancer Foundation. This work was sponsored by operating grants from the Cancer Research Society and the Canadian Cancer

Society Research Institute (formerly the Canadian Breast Cancer Research Alliance). We thank Ann Tollefson (Saint Louis University School of Medicine) for LCRT cells and Dr. Kathleen Delaney and Marion Corrick for technical assistance with cotton rat housing and sedation.

Disclosures:
The authors acknowledge there are no financial conflicts of interest related to this research.

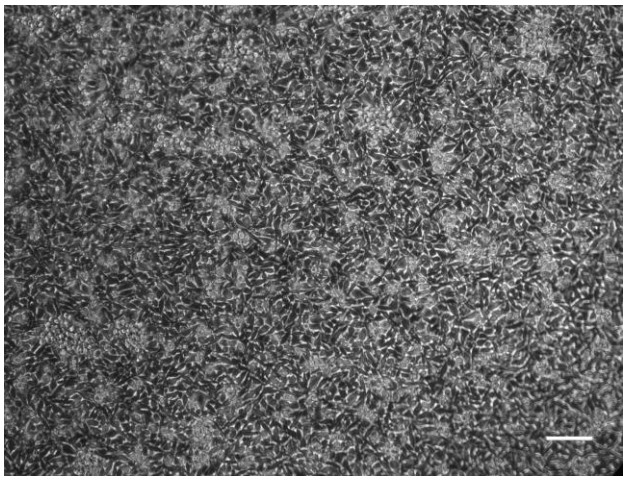
References:
1 Cervantes-Garcia, D., Ortiz-Lopez, R., Mayek-Perez, N. & Rojas-Martinez, A. Oncolytic virotherapy. *Ann Hepatol.* **7** (1), 34-45, (2008).
2 Vaha-Koskela, M. J., Heikkila, J. E. & Hinkkanen, A. E. Oncolytic viruses in cancer therapy. *Cancer Lett.* **254** (2), 178-216, doi:10.1016/j.canlet.2007.02.002, (2007).
3 Abril, C. *et al.* Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. *J Virol.* **78** (7), 3644-3653, doi: 10.1128/JVI.78.7.3644-3653.2004, (2004).
4 Nakamichi, K., Matsumoto, Y. & Otsuka, H. Defective infection of bovine herpesvirus 1 in non-permissive murine cells. *J Vet Med Sci.* **63** (10), 1139-1142 (2001).
5 Boukhvalova, M. S. & Blanco, J. C. The cotton rat sigmodon hispidus model of respiratory syncytial virus infection. *Curr Top Microbiol Immunol.* **372** 347-358, doi:10.1007/978-3-642-38919-1_17, (2013).
6 Papp, Z., Babiuk, L. A. & Baca-Estrada, M. E. Induction of immunity in the respiratory tract and protection from bovine herpesvirus type 1 infection by different routes of immunization with recombinant adenovirus. *Viral Immunol.* **11** (2), 79-91, doi:10.1089/vim.1998.11.79, (1998).
7 Hughes T, C. R., Lilley CE, Ponce R, Kaufman HL. Critical analysis of an oncolytic herpesvirus encoding granulocyte-macrophage colony stimulating factor for the treatment of malignant melanoma. *Journal of Oncolytic Virotherapy.* **3** 11-20, doi:10.2147/OV.S36701, (2014).
8 Jones, C. & Chowdhury, S. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev.* **8** (2), 187-205, doi:10.1017/S1466252307001340X, (2007).
9 Jones, C. & Chowdhury, S. Bovine herpesvirus type 1 (BHV-1) is an important cofactor in the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract.* **26** (2), 303-321, doi:10.1016/j.cvfa.2010.04.007, (2010).
10 Hushur, O., Takashima, Y., Matsumoto, Y. & Otsuka, H. Restriction of bovine herpesvirus 1 (BHV-1) growth in non-permissive cells beyond the expression of immediate early genes. *J Vet Med Sci.* **66** (4), 453-455, doi: 10.1292/jvms.66.453, (2004).
11 Cuddington, B. P., Dyer, A. L., Workenhe, S. T. & Mossman, K. L. Oncolytic bovine herpesvirus type 1 infects and kills breast tumor cells and breast cancer-initiating cells irrespective of tumor subtype. *Cancer Gene Ther.* **20** (5), 282-289, doi:10.1038/cgt.2013.18, (2013).
12 Cuddington, B. P. & Mossman, K. L. Permissiveness of Human Cancer Cells to Oncolytic Bovine Herpesvirus 1 Is Mediated in Part by KRAS Activity. *J Virol.* **88** (12), 6885-6895, doi:10.1128/JVI.00849-14, (2014).

13 Small, E. J. *et al.* A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. *Mol Ther.* **14** (1), 107-117, doi:10.1016/j.ymthe.2006.02.011, (2006).
14 Freytag, S. O. *et al.* Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.* **62** (17), 4968-4976 (2002).
15 Benjamin, R., Helman, L., Meyers, P. & Reaman, G. A phase I/II dose escalation and activity study of intravenous injections of OCaP1 for subjects with refractory osteosarcoma metastatic to lung. *Hum Gene Ther.* **12** (12), 1591-1593 (2001).
16 Prince, G. A. The Cotton Rat in Biomedical Research. *Animal Welfare Information Center Newsletter.* **5** (2), 3-5, http://www.nal.usda.gov/awic/newsletters/v5n2/5n2princ.htm, (1994).
17 Tsai, J. C., Garlinghouse, G., McDonnell, P. J. & Trousdale, M. D. An experimental animal model of adenovirus-induced ocular disease. The cotton rat. *Arch Ophthalmol.* **110** (8), 1167-1170, doi:10.1001/archopht.1992.01080200147043, (1992).
18 Ginsberg, H. S. *et al.* A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. *Proc Natl Acad Sci U S A.* **88** (5), 1651-1655 (1991).
19 Russell, S. J., Peng, K. W. & Bell, J. C. Oncolytic virotherapy. *Nat Biotechnol.* **30** (7), 658-670, doi:10.1038/nbt.2287, (2012).
20 Mossman, K. L., Saffran, H. A. & Smiley, J. R. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. *J Virol.* **74** (4), 2052-2056, doi: 10.1128/JVI.74.4.2052-2056.2000, (2000).
21 Mossman, K. L. & Smiley, J. R. Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. *J Virol.* **76** (4), 1995-1998, doi: 10.1128/JVI.76.4.1995-1998.2002, (2002).
22 Hummel, J. L., Safroneeva, E. & Mossman, K. L. The role of ICP0-Null HSV-1 and interferon signaling defects in the effective treatment of breast adenocarcinoma. *Mol Ther.* **12** (6), 1101-1110, doi:10.1016/j.ymthe.2005.07.533, (2005).
23 Papp, Z., Middleton, D. M., Mittal, S. K., Babiuk, L. A. & Baca-Estrada, M. E. Mucosal immunization with recombinant adenoviruses: induction of immunity and protection of cotton rats against respiratory bovine herpesvirus type 1 infection. *J Gen Virol.* **78 (Pt 11)** 2933-2943 (1997).
24 Papp, Z., Babiuk, L. A. & Baca-Estrada, M. E. The effect of pre-existing adenovirus-specific immunity on immune responses induced by recombinant adenovirus expressing glycoprotein D of bovine herpesvirus type 1. *Vaccine.* **17** (7-8), 933-943, doi:10.1016/S0264-410X(98)00279-5, (1999).
25 Mittal, S. K. *et al.* Induction of systemic and mucosal immune responses in cotton rats immunized with human adenovirus type 5 recombinants expressing the full and truncated forms of bovine herpesvirus type 1 glycoprotein gD. *Virology.* **222** (2), 299-309, doi:10.1006/viro.1996.0427, (1996).
26 Steel, J. C. *et al.* 322. Syngeneic Cotton Rat Cancer Model for Replicating Adenoviral Vectors. *Molecular Therapy.* **13** (S1), S123, doi: 10.1016/j.ymthe.2006.08.379, (2006).
27 Toth, K. *et al.* Cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Hum Gene Ther.* **16** (1), 139-146, doi:10.1089/hum.2005.16.139, (2005).

28 Toth, K., Spencer, J. F. & Wold, W. S. Immunocompetent, semi-permissive cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Methods Mol Med.* **130** 157-168, doi: 10.1016/j.virol.2007.07.022, (2007).
29 Steel, J. C. *et al.* Immunocompetent syngeneic cotton rat tumor models for the assessment of replication-competent oncolytic adenovirus. *Virology.* **369** (1), 131-142, doi:10.1016/j.virol.2007.07.022, (2007).
30 Workenhe, S. T. *et al.* Immunogenic HSV-mediated oncolysis shapes the antitumor immune response and contributes to therapeutic efficacy. *Mol Ther.* **22** (1), 123-131, doi:10.1038/mt.2013.238, (2014).
31 Sobol, P. T. *et al.* Adaptive antiviral immunity is a determinant of the therapeutic success of oncolytic virotherapy. *Mol Ther.* **19** (2), 335-344, doi:10.1038/mt.2010.264, (2011).
32 Prince, G. A. The Cotton Rat in Biomedical Research. *Animal Welfare Information Center Newsletter.* **5** (2), http://www.nal.usda.gov/awic/newsletters/v5n2/5n2princ.htm, (1994).

Figure
Click here to download Figure: Figure 1.pdf

A)



B)

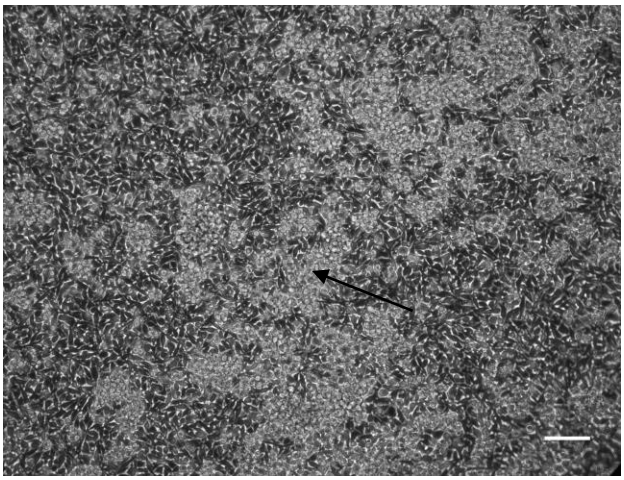


Figure
Click here to download Figure: Figure 2.pdf

Figure
Click here to download Figure: Figure 3.pdf

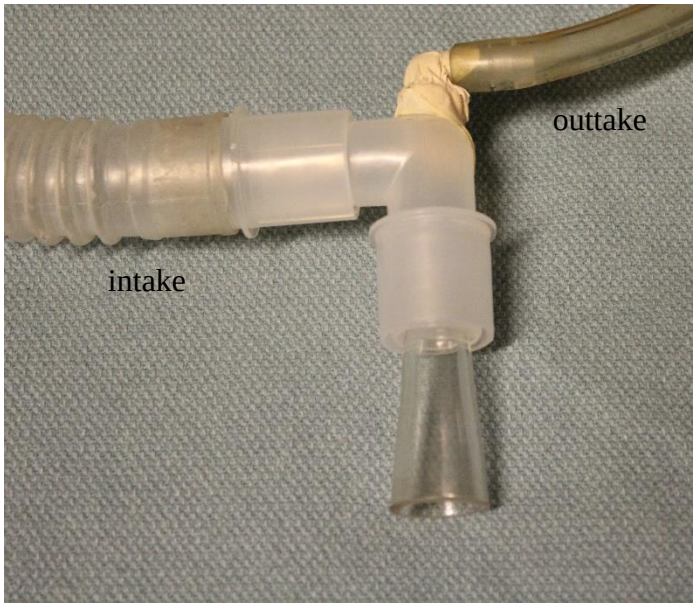
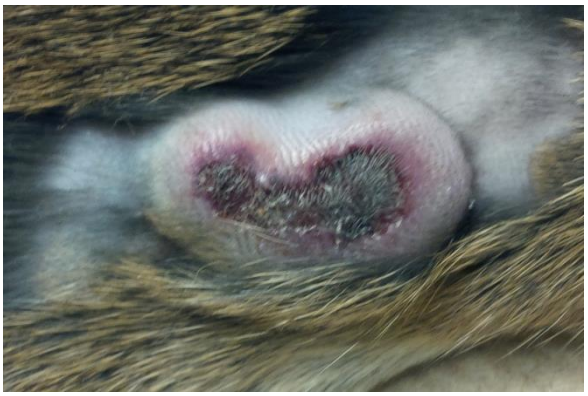
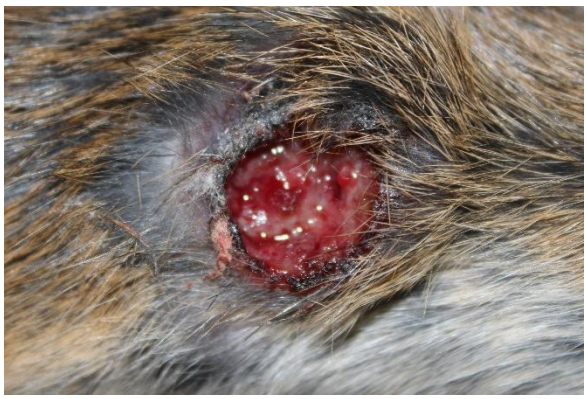


Figure
Click here to download Figure: Figure 4.pdf

A)



B)



Figure

Click here to download Figure: Figure 5 Updated.pdf

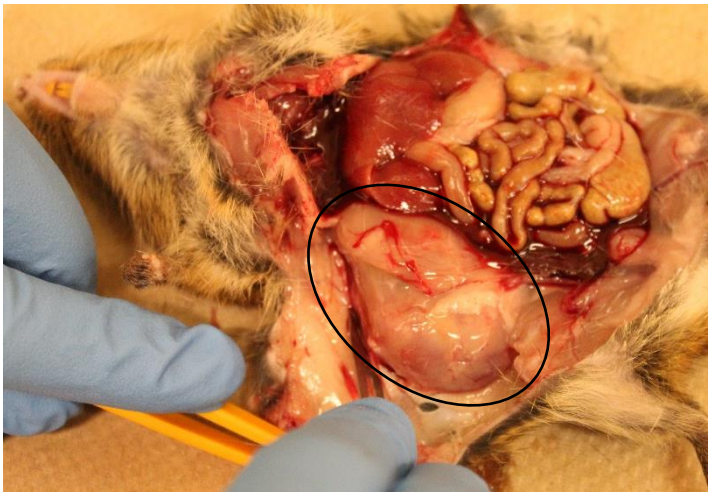
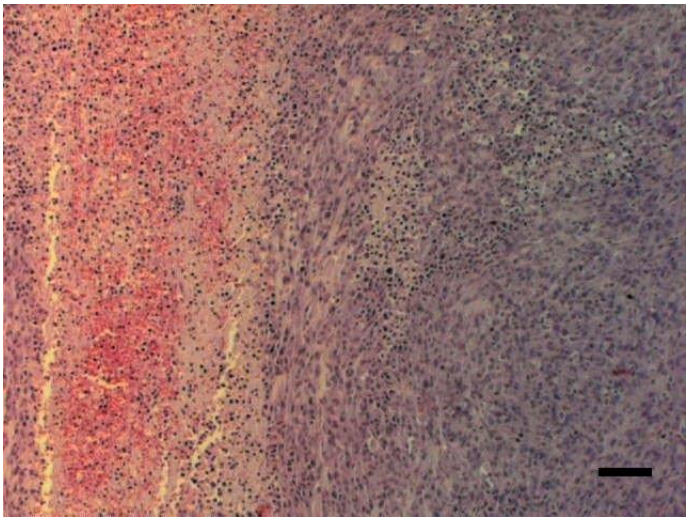


Figure
Click here to download Figure: Figure 6.pdf



Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Materials and Equipment.xls

Name of Material/Equipment	Company	Catalog #
Dulbecco's modified Eagle's medium	Gibco	11965-092
1X Phosphate Buffered Saline		
200 mM L-glutamine	Gibco	25030164
100x Antibiotic-Antimycotic	Gibco	15240-062
Fetal bovine serum	Quality Biological Inc.	110-001-101HI
T-150cm^2 tissue culture flask	Fisher Scientific	14-826-80
1X TypLE Express	Life Technologies	12604-013
12-well cell culture plate, flat bottom	Fisher Scientific	08-772-29
alamarBlue	Life Technologies	DAL1025
8640 Teklad 22/5 Rodent diet	Harlan	8640
1/8" corncob rodent bedding	Harlan	7092
Nestlets	Ancare	-
50 mL Conical tubes	Fisher Scientific	14-432-22
Isoflurane USP, 99.9 %, inhalation anesthetic	Pharmaceutical Partners of Canada Inc.	M60302
70% Ethanol		
10 % Neutral Buffered Formalin	Sigma-Aldrich	HT501128

Comments/Description
May use any brand
Can prepare in lab, filter to sterilize
May use any brand
May use any brand
May use any brand
May use any brand
May use any brand, must be tissue culture treated
May use an alternative reagent for determination of cell viability
Made of pulped virgin cotton fiber, dust-free and autoclavable
May use any brand, must be sterile
Can prepare in lab
May use any brand

Name of Material/Equipment	**Company**	**Catalog #**
NAPCO NapFlow 1200 Class II A/B3 Biosafety Microbiological Safety Cabinet (cell culture hood)	NAPCO	Model used not currently available
Thermo Fisher Scientific Precision Heated Water Bath	Fisher Scientific	Model used not currently available
Reichert Bright-line Hemacytometer	Sigma-Aldrich	Z359629
Typhoon Trio BioAnalyzer	GE Healthcare Life Sciences	Model used not currently available
Tecan Safire^2 Multi-detection Microplate Reader	Tecan	Model used not currently available
Allegra 6R benchtop centrifuge	Beckman Coulter	366816
Table Top Anaesthesia machine	VetEquip	Model used not currently available
Wahl Peanut Mini Clippers	Wahl	
Insulin syringes 29 G x 1/2', 0.3 mL	BD	329464
Cotton swabs	MedPro	018-425
Sharp-Pointed Dissecting Scissors	Fisher Scientific	8940
Dissecting Tissue Forceps	Fisher Scientific	13-812-41

Comments/Description
May use any brand
May use any brand
May use any brand
May use any fluorescence plate reader
May use any fluorescence plate reader
May use any brand
May use any brand, must be portable
May use any brand of small clippers
May use any brand. Insulin syringes are recommended as they make injections easier through the rat's tough skin.
May use any brand
May use any brand
May use any brand

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: **Cotton rats as a model for the pre-clinical evaluation of oncolytic viruses**

Author(s): Breanne Cuddington, Dr. Meghan Verschoor and Dr. Karen Mossman

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Dr. Karen Mossman
Department:	Pathology and Molecular Medicine
Institution:	McMaster University
Article Title:	**Cotton rats as a model for the pre-clinical evaluation of oncolytic viruses**
Signature:	 Date: **April 18, 2014**

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Rebuttal Comments
Click here to download Rebuttal Comments: Rebuttal letter (JOVE).doc

Dr. Jaydev Upponi
Science Editor
Journal of Visualized Experiments

June 30, 2014

Dear Dr. Upponi,

Thank you for handling our manuscript. We appreciate the efforts of yourself and the reviewers. All concerns outlined by the four reviewers have been addressed. Please find below comments from the reviewers (copied and in bold) along with our response to each point raised. Changes are tracked within the manuscript. The introduction was rewritten to address the comments raised by reviewer # 1 with the revised version inserted into the manuscript.

Editorial comments:

1) Section 1 should start with 1.1 and not 1.0

Sections numbers have been amended as per requested.

2) Type of cell should be included in step 2.1.1.

The point now includes mention of the specific cell line used, LCRT.

3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

The manuscript has been carefully edited for spelling and grammar issues.

4) Please note that reviewers # 4 has raised some serious concerns about some aspects of your manuscript. Please thoroughly address or rebut each comment to further strengthen and clarify your submission.

The concerns raised by reviewer #4 have been addressed. Although serious concerns were raised, we strongly feel that this submission would greatly contribute to ease of use of this model.

5) Please disregard this comment if all of your figures are original. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures are original.

We would like to take the opportunity to address the major concerns posed by reviewers pertaining to the relevance of this manuscript given the availability of other published studies on the use of cotton rats as a pre-clinical model for the evaluation of oncolytic viruses. These concerns have been amalgamated below, followed by our rebuttal.

Reviewer #1:

3. Much of the information about handling of the cotton rat has been previously described, including the use of the enrichment tube to move the cotton rat to the anaesthesia machine (Ayers et al. J Am Assoc Lab Anim Sci. 2012). Excising tumours for histology or images of necrotic tumours are not new either. So the question is what new does this manuscript bring to work on cotton rats? Showing figures with the growth curves of LCRT, or anti-tumor effects or something related to the evaluation of oncolytic viruses in the cotton rat would have been more constructive and would add to the manuscript.

Reviewer #4:

The manuscript is largely based on a previously published methods chapter by Toth et al. (Toth K., Spencer J.F., Wold W.S.M. Immunocompetent, Semi-permissive Cotton Rat Tumor Model for the Evaluation of Oncolytic Adenoviruses in: Adenovirus Methods and Protocols, Second Edition. Eds.: Tollefson, A.E., and Wold, W.S.M., 157-68, 2007). Although the authors have added several novel procedures, the bulk of the manuscript is a close reproduction of the article by Toth et al., which is not referenced by the authors.

When we initially considered the cotton rat as an *in vivo* model to study the antitumor efficacy of BHV-1, we performed an extensive literature search and contacted researchers that have experience with these animals. Although several publications exist on the handling, housing and methods to evaluate oncolytic viruses using this model, there were discrepancies. For example, both individual and co-housing of the cotton rats were suggested [1,2]. In our experience, individual housing decreases stress to the animals and the researcher, as well as facilitating capture. Although scruffing was described as a relevant method by which to restrain the rats [1,3], this is not advisable due to the extreme flight or fight response of the animals and the high likelihood of causing trauma to the cotton rat from it biting its tongue and bleeding from the mouth. Furthermore, researchers familiar with mice and conventional rat species may find scruffing difficult or impossible. Unless the researcher has experience performing wild rodent captures, alternative capture methods to scruffing are required. The paper by Ayers and colleagues describes capture of cotton rats in their enrichment tubes with direct transportation to the anesthesia induction chamber [4]. Unfortunately, during our studies and method development, we were unaware of this publication. However, this highlights the utility of a comprehensive methods paper that includes a video portion to instruct researchers on the proper handling techniques to minimize stress to the rats. This manuscript and video will also prepare individuals for the behavioral challenges in working with cotton rats. It is not uncommon to encounter misnomers about the behaviour of cotton rats, which are often mistaken with that of conventional laboratory rat species. Furthermore, videos are unavailable to provide researchers with a visual

account of the excitable nature of cotton rats. This visual representation of the handling techniques is meant to complement written accounts (such as those referenced by the reviewers) to better prepare researchers for the challenges faced with this model, as we strongly feel that written accounts fail to adequately describe the nature and characteristics of cotton rats.

Reviewers' comments:

Reviewer #1:

This manuscript describes the special handling techniques used to work with the cotton rat particularly in relation to evaluating oncolytic viruses. The authors also describe the growth and implantation of a tumour line (LCRT) and its use in this animal model. The manuscript is generally well written, with the protocols well explained and presented in a way to allow replication of the work. This being said, I have a number of concerns with the manuscript:

Major Concerns:

1. The manuscript fails to describe how the model can be used for the evaluation of oncolytic viruses, and this is the title of the paper.

There is a brief description of culturing of LCRT cells in vitro and some minor information about how to check if the virus (which virus used is not described at all) transduces these cells in culture but there is no information about how to assess this in the cotton rat. Further, there is no information about how to assess oncolysis/replication of the virus in the cotton rat which should be a major component of this manuscript given the title.

The title of the manuscript has been changed to reflect its primary purpose; to provide readers with detailed information pertaining to the handling, care and how to perform procedures on cotton rats while minimizing risk to the animals and the researcher.

2. There is a fair amount of the introduction dedicated to BHV-1 and HSV-1 but neither of these viruses have been shown to replicate in the LCRT cell line. BHV-1 does not replicate in human cells so I do not see the relevance of this as an oncolytic virus or even a target for an oncolytic virus. The LCRT cell line does allow for the assessment of oncolytic adenovirus, as has been described by at least 2 groups. The introduction needs to be re-written to discuss the relevant vector for the evaluation of Oncolytic virus in LCRT, i.e. adenovirus.

The introduction has been rewritten to focus on the development of the cotton rat as an alternative model for oncolytic viruses for which conventional murine models are not feasible. The introduction provides two examples, BHV-1 and adenovirus vectors, as oncolytic viruses currently under pre-clinical evaluation which would benefit from the cotton rat model. References to specific studies that describe the oncolytic capacity of BHV-1 in multiple human tumor cell types have been added to the introduction.

Minor Concerns:

1. In the representative results section the authors suggest using a larger needle for injection of tumour cells. This is not described in the methods, nor an example for what size

needle to use.

The size of needle (29 G x 1/2', 0.3 mL) has been added to the appropriate methods sections.

Reviewer #2:

Manuscript Summary:

The authors describe techniques for handling cotton rats, which are highly excitable. The techniques stress maximizing safety and minimizing stress of both the researcher(s) and rats. The manuscript progresses to describe how to grow and implant mammary fibrosarcoma cells for the purpose of testing immunotherapies such as oncolytic virotherapy. Guidelines for necropsies and excising tumors are also provided.

We thank the reviewer for their positive comments and comprehensive summary of the study presented.

Major Concerns:

In the introduction the authors mention that cotton rats are used as a model of BHV-1 respiratory infection. They also discuss BHV-1 in the context of oncolytic virotherapy by comparing it to the KM100 HSV-1 oncolytic virus. Do the authors plan on testing BHV-1 as an oncolytic agent in cotton rats. If so, are there safety concerns with respect to its potential to cause respiratory disease?

Studies are being conducted in our laboratory using the cotton rat model of breast adenocarcinoma to evaluate the in vivo oncolytic capacity of BHV-1. Given the ability of BHV-1 to infect and induce cytotoxicity in cotton rat cells, particularly in the lung, care has been taken to monitor animals for respiratory distress. In anti-BHV-1 vaccination studies, the standard challenge dose is 10^7 pfu BHV-1 delivered intranasally. To mitigate any potential respiratory distress by the virus, 10^7 pfu is being considered the maximal tolerated dose for use in our experiments. Indeed, our current studies suggest that even at this dose, there is no evidence of virus replication within the lung.

The methods describe working with LCRT cells and using them to establish mammary fibrosarcomas. Why were these cells injected subcutaneously rather than into the mammary fat pad?

LCRT cells were injected subcutaneously to ease the delivery of treatment(s) (BHV-1 or another oncolytic virus in this case) so they can be administered in a timely manner to minimize long-term exposure of the cotton rats to anesthesia. Sedation of cotton rats for extended periods of time is not recommended and their ability to recover from sedation decreases with each incidence.

Line 357: intratumoral injections were performed by moving the bevel of the needle in a fan-like pattern. This would severely lacerate the tumour. A more typical approach would be to partially withdraw the needle and then re-insert it into a different part of the tumour and to do this repeatedly in an attempt to achieve complete three-dimensional coverage.

The method used for intratumoral injections requires clarification, which has been added to the manuscript. The needle was moved in a fan-like pattern but with a partial withdraw preceding each movement to prevent laceration of the tumor. This was used to achieve as complete coverage as possible with the treatment.

Generally, the methods seemed to be written in a format reminiscent of personal-use protocols. Some key details are missing, there are grammatical errors, equations are confusing/incorrect, etc. See the numerous specific comments below.

Minor Concerns:
General comments:
These comments are based on "revision 1".

A typical and strong recommendation when working with rats is to handle them daily for at least one week prior to initiation of experiments so that they can accommodate to their handler. This significantly calms most rats. Do cotton rats respond to this type of accommodation?

Handling of the cotton rats prior to experiments was not done by the specific researcher performing the experiments. An animal facility technician handles the animals when changing their cage, food and water as well as doing health checks on a daily basis. However, we found that performing procedures after handling by the technician was more difficult with the animals being more easily excitable and agitated.

There is reference to LCRT-derived tumors being a "fibrosarcoma of the breast". Mice don't have breasts. Therefore, these are mammary fibrosarcomas.

Thank you for the clarification, this change has been made to the manuscript.

Figures are presented in the text in the following order: Fig. 1, 2, 3, 5, 6, 4. Therefore, the figures should be switched to account for this sequence.

The figures in the text are now in sequential order and coincide with the appropriate legend.

Section 7.5-7.7: The authors make reference to potential metastases. Do they have any images that they could provide as examples (especially of lymph nodes that are enlarged and/or with metastases, since these can be a challenge to locate)?

Metastases are primarily found in the armpit on the same side as the primary tumor as well as posterior to the primary tumor. These metastases grow quickly and are easily detected by palpation. Lung metastases are also very common, but are difficult to detect until their growth results in breathing difficulty by the animal. Unfortunately, images of metastases prior to excision are unavailable.

Reference is made several times to the need to "stay calm" when using cotton rats. This implies that the rats can sense the emotional status of the handler. Indeed, this is stated in

lines 499-500. Is there a reference with objective data to support this or is it a subjective assessment by the authors?

This is a subjective assessment based on the experience of the researchers over multiple experiments. These statements are drawn from observed differences in the demeanor of the cotton rats following such events as previous handling by animal facility technicians, failed trapping attempts and escapes.

The authors mention that LCRT, CCRT and VCRT cells can be used in cotton rats and specify that the latter are used as models of osteosarcoma but all of the cell-specific methods that are described are for the LCRT cells only. This emphasis should be clearly stated in both the introduction and abstract. For example, the intratumoral injections presumably wouldn't work well in the context of bone cancers.

The specific focus of the manuscript on the cotton rat model of mammary carcinoma has been further emphasized in the abstract and introduction. The introduction has been expanded to discuss studies evaluating oncolytic herpesviruses in multiple models of mammary carcinoma. Furthermore, the evaluation of BHV-1 as an oncolytic in human breast tumor cells has been added, as well as justification for the use of the cotton rat model to study its *in vivo* efficacy due to issues with using conventional murine models. These additions serve to emphasize the focus of this manuscript on the cotton rat model of mammary carcinoma.

Specific comments:

Line 113: "culture until confluent", which implies 100% confluency; then states 90% confluency - which one is it?

This point has been clarified.

Line 115: what is the concentration of the trypsin?

The concentration of the trypsin is 1X. This has been added to the manuscript.

Line 136: can the authors provide an example of a plate size they used and how many cells at the time of seeding resulted in 80-90% confluency one day later?

A concentration of 10^5 cells/mL (100 µL per well) was used to produce 80-90% confluent monolayers one day later in 96-well flat bottom plates for virus replication experiments. These data have been added.

Line 151: "prior to charge" = awkward wording

This wording could not be found. The section was carefully checked for grammar.

Line 155-156: this equation is very confusing and could be expressed more logically. In line 156 there are multiple references to volumes, but volumes of what? Why not calculate the

required # of pfu's of virus = # of plated cells x MOI, and then calculate the volume of stock virus required = # of pfu needed / virus titer (pfu/ml)?
The required changes were made.

Line 159: why is it important for serum-free medium to be used to prepare the virus inoculum?

The use of serum-free medium reduces the risk of biological contaminants contained in fetal bovine serum from interfering with the virus infection.

Lines 168 and 173: Are these the same solutions?

Yes, this has been clarified.

Line 173: "AlamarBlue" as a single word is a trade name; perhaps the generic name would be better?

AlamarBlue has been changed to resazurin.

Line 176: need to define the abbreviations "ex" and "em".

Abbreviations have been defined.

Line 178: What is background fluorescence? Is it medium only?

The control for background fluorescence is a well of medium only. This has been added.

Line 211: "hand" = "handle"?

Amended.

Line 213: "over" should be "instead of"?

Amended.

Line 222: "induction chamber" = anesthetic induction chamber?

Anesthetic has been added to clarify the type of chamber.

Line 226: the authors should clarify that isoflurane is an anesthetic

Isoflurane has been clarified as an inhalation anesthetic.

Line 233: awkward wording; implies the tube itself is to travel through the bars

Phrase has been re-worded to avoid confusion.

Line 256: delete "handler #2" - it is confusing having this with "handler 1" immediately after it.

Deleted “handler 2”.

Line 268: light mineral oil/white petrolatum is the generic name

The name used indicates a generic ophthalmic ointment that has a petrolatum base. Petralube vet ointment is the specific name.

Line 274: how is the heart rate monitored? Would monitoring respiratory rate be easier? What is an acceptable rate?

The animal’s respiration rate was monitored during all procedures and was maintained by adjusting the flow of isoflurane. The respiration rate should be kept constant during all procedures. This has been clarified in the manuscript.

Line 283: delete "per animal"? The # is based on the total cells needed for the experiment.

Deleted.

Line 285: "preparation and preparation" = redundancy

Deleted.

Line 285-286: avoid using double negatives (i.e. "reduce decreases" = increase).

Double negative has been replaced with “to prolong cell viability”.

Line 311-312: this equation doesn't make sense; "cells required" = per mouse, for all mice or for all mice + extra doses? Dividing by the total cell count means the more cells there are, the lower the volume will be; but the opposite is needed (i.e. more cells requires a larger volume to adequately dilute them).

The equation has been amended to calculate for the total volume of cell suspension required to inject all animals in the experiment. This will provide a larger volume in which to adequately dilute the cells.

Line 314: What about CCRT and VCRT cells?

This methods paper deals specifically with the cotton rat model of mammary carcinoma. This has been emphasized in the abstract and introduction.

Line 317-318: It would be better to dilute all of the cells in the same tube to the desired concentration to minimize the variability introduced by multiple pipetting. Is 100

microliters the desired injection volume? If so, shouldn't the volume be >100 microliters to account for losses and to avoid dulling needles by having them contact the bottom of the microtube?

The calculation and subsequent steps have been amended to describe preparation of a master mix which will be used to prepare all injections.

Line 332-334: the ethanol should be allowed to evaporate completely since it inactivates viruses

The stipulation to allow the EtOH to evaporate completely before proceeding has been added to the necessary steps.

Line 337: should include a statement that the needle needs to be in an upright position.

This has been added to the step.

Line 340: this procedure is typically referred to as "skin tenting"

The term skin tenting has been added to this step.

Line 343-344: what is the rationale for inserting the needle bevel side up and withdrawing it bevel side down?

Syringes are designed to be inserted bevel side up to ease intradermal injection by allowing the sharpest point of the needle to contact the skin first. This also reduces the pain from injection. The needle is withdrawn bevel side down to ensure maximal delivery of the treatment.

The beginning of sections 6.1, 6.2 and 6.3 are identical; it would significantly reduce redundancy if these were fused into one section.

The beginning of sections 6.1, 6.2 and 6.3 are similar, but not identical. Moreover, subsequent steps describe important differences between these injection techniques that may not be obvious to trainees or those new at injection practices. For this reason we would like to keep these sections separate.

Line 356: Where is the needle being inserted? …into the tumor? Where is the tumor located (there are references to both subcutaneous and intraperitoneal injection sites)?

In this case, the needle is being inserted into a subcutaneous tumor. Clarification has been provided at the beginning of the section emphasizing that this section pertains to intratumoral injection into subcutaneous LCRT tumors.

Earlier in the paper, reference was made to osteosarcomas; will this method work for these?

While we do not have personal experience with the cotton rat model of osteosarcoma, there are several published studies evaluating the *in vivo* oncolytic capacity of adenovirus-based vectors in this model. These studies involve subcutaneous injection of the osteosarcoma cotton rat cell lines VCRT and CCRT to establish tumors that were then treated by intratumoral injection [5,6]. Therefore, we do not feel that there would be significant challenges in applying the methods described to a cotton rat model of osteosarcoma.

Line 359: what is the rationale for doing i.p. injections?

Intraperitoneal injections are commonly used to delivery chemotherapeutics in combination studies with oncolytic viruses. Therefore, we felt that this method should be included.

Line 370: feces of normal consistency can't be aspirated with a 29G needle. [Not] aspirating blood indicates [correct] placement of the needle.

An amendment stating that not aspirating blood indicates correct placement of the needle has been added.

Line 376: what tools are required?

Sharp-pointed dissection scissors, dissecting tissue forceps and a scalpel. These materials are listed in the materials and equipment table.

Line 402: The "small" and "large" tumors should be defined by some objective parameters.

A small tumor section is considered anything equal to or less than 2 mm in width and should be fixed in 10% NBF for 1-2 days. A larger tumor (greater than 2 mm) should be fixed for 5-6 days.

Line 406: "…necrotic and hemorrhagic [centers] often form on the [surface]…" The bracketed terms seem contradictory.

This has been edited to omit the contradictory terms.

Line 411: grammatical error: "…ease in their use as…"

No grammatical error was found on this line.

Line 426: "…obtain discard animals to practise injection techniques prior to attempting them on the rats." How are "discard animals" defined? Are they a species other than rats as is implied by the wording?

Discard animals are those not required by other researchers for experiments and would otherwise be euthanized. These animals can be of any species as they serve to familiarize the researcher with general injection techniques. The operational definition of “discard animal” has been clarified in the text.

Line 429: It would be helpful to recommend a needle gauge size in the methods section.

The size of the needle gauge has been added to the appropriate sections.

Line 439: grammar: "often" is repeated

Repetition amended.

Line 451: "effect" should be "affect"

Change made.

Line 504: Why is extended sedation problematic in cotton rats?

Extended sedation is problematic as it increases the recovery time of the animals. Specifically, the time taken for them regain consciousness and motor control increases the longer they are exposed to anesthesia. The long term effects on the animals and how it may impact results is unclear.

Line 522: Is it the phenobarbital that directly preserves the tumor tissues or the form of euthanasia? The wording can be interpreted to mean the former.

Phenobarbital was not used to preserve tumor tissues. In order to preserve the tissues for analysis the necropsy was performed immediately following euthanization by CO_2.

Line 534: presumably the "research" does not experience stress or injury.

“Research” has been corrected to “researcher”.

Line 535: the use of the word "particularly" seems odd. Is there a reason why the model would serve oncolytic virotherapy-focused research better than other types of immunological studies? Perhaps "including" would be more appropriate.

“Particularly” has been changed to “including”.

Line 541: Is the reference to the now defunct "Canadian Breast Cancer Research Alliance" required (or accurate)? The CCS was just one of many partners that comprised the former alliance.

The funding provided for these studies was granted under the Canadian Breast Cancer Research Alliance. Reference to the CBCRA is in accordance with the wishes of the funding partner.

Figure 5: it would be very helpful to draw a circle around the tumor to make its location more obvious to those without necropsy experience.

A circle has been drawn around the tumor to highlight its location.

Reviewer #3:

Manuscript Summary:

Oncolytic viruses have been developed as a new class of cancer therapeutic agents for treatment of cancer that are resistant to conventional therapies. There have been increasing evidence that the antitumor efficacy by oncolytic viruses depends on the interaction of tumour cells, viruses and host immune response. Using immunocompetent animal models will be more meaningful for validation of efficacy and safety of oncolytic viruses. Cotton rat is one of immunocompetent model for this purpose. This manuscript describes the proper handling procedure for this useful model and how to establish SC tumour models in Cotton Rats. This will be of high interest and benefit to the researchers in the field of cancer virotherapy, its worthy to be published. However, the quality could be further improved if the authors appreciate my comments below.

We thank the reviewer for their positive comments and comprehensive summary of the study presented.

Major Concerns:

1. Considering the availability of tumour models, research tools and handling issues etc, mice might be more popular, the authors should beef up what advantages of Cotton rat has as a model for assessment of oncolytic virus? such as mouse tissue and cells do not support replication of some oncolytic viruses, for example, adenovirus; In addition host immune responses to some viruses in Cotton Rat might be **more closed to human than mouse?**

The rationale for using the cotton rat as a model for the pre-clinical evaluation of oncolytic viruses has been emphasized in the introduction, specifically pertaining to BHV-1 and oncolytic adenovirus vectors, for which conventional murine models cannot be used. Furthermore, advantages of using immunocompetent tolerized models for antitumor studies have also been added. We hope that this emphasis will add to the argument for cotton rats as a relevant *in vivo* model to evaluate the antitumor efficacy of oncolytic viruses.

2. Some oncolytic viruses are permissive in mouse normal tissues and tumour cells, the authors should address what kind of oncolytic viruses should be validated using Cotton Rats or other models? Such as adenovirus, if discuss this, the author should also discuss what the advantages of **Cotton rat has compared to Syrian hamster model which supports adenovirus replication very well.**

A thorough discussion on the advantages of using the cotton rat as a model for the pre-clinical evaluation of oncolytic adenovirus vectors has been included in the introduction. Furthermore, as the repertoire of oncolytic viruses increases so does the demand for relevant models to test their antitumor efficacy. In the case of adenovirus and BHV-1 (and other not yet described oncolytic viruses), this does not include conventional murine models. Therefore, the development of the cotton rat and other alternative tumor models, such as the Syrian hamster, will aid in advancements in the field of oncolytic virotherapy.

Minor Concerns:

It would be very useful that authors can make a table to list all established tumour cell lines derived from Cotton Rat in the second part. This will be very informative for the readers.

There exist three cotton rat tumor cell lines derived from spontaneous fibrosarcoma and osteosarcomas of the mammary gland (LCRT) and bone (CCRT and VCRT), respectively. These cell lines are listed in the introduction with subsequent methods focused on the use of LCRT cells to establish a cotton rat model of subcutaneous mammary carcinoma for the pre-clinical evaluation of oncolytic viruses.

Reviewer #4:

Manuscript Summary:

The authors describe techniques to evaluate oncolytic (replication-competent) virus vectors in cotton rats. They describe culturing a cotton rat cell line, generating tumors in the animals, and assessing the effect of the vector on the tumor. Further, they give instructions for handling this difficult to work with rodent species. The manuscript describes the procedures in adequate details.

We thank the reviewer for their comments and positive feedback regarding the details provided in this manuscript.

Major Concerns:

The manuscript is largely based on a previously published methods chapter by Toth et al. (Toth K., Spencer J.F., Wold W.S.M. Immunocompetent, Semi-permissive Cotton Rat Tumor Model for the Evaluation of Oncolytic Adenoviruses in: Adenovirus Methods and Protocols, Second Edition. Eds.: Tollefson, A.E., and Wold, W.S.M., 157-68, 2007). Although the authors have added several novel procedures, the bulk of the manuscript is a close reproduction of the article by Toth et al., which is not referenced by the authors.

The article by Toth and colleagues (Toth K., Spencer J.F., Wold W.S.M. Immunocompetent, Semi-permissive Cotton Rat Tumor Model for the Evaluation of Oncolytic Adenoviruses in: Adenovirus Methods and Protocols, Second Edition. Eds.: Tollefson, A.E., and Wold, W.S.M., 157-68, 2007) is reference #28 in the manuscript and is cited in the introduction.

Minor Concerns:

N/A

References

1 Toth, K., Spencer, J. F. & Wold, W. S. Immunocompetent, semi-permissive cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Methods in molecular medicine* **130**, 157-168 (2007).

2 Ward, L. E. Handling the cotton rat for research. *Lab animal* **30**, 45-50 (2001).

3 Prince, G. A. The Cotton Rat in Biomedical Research. *Animal Welfare Information Center Newsletter* **5**, 3-5 (1994).

4 Ayers, J. D., Rota, P. A., Collins, M. L. & Drew, C. P. Alternatives to retroorbital blood collection in hispid cotton rats (Sigmodon hispidus). *Journal of the American Association*

for Laboratory Animal Science : JAALAS **51**, 239-245 (2012).

5 Steel, J. C. *et al.* Immunocompetent syngeneic cotton rat tumor models for the assessment of replication-competent oncolytic adenovirus. *Virology* **369**, 131-142, doi:10.1016/j.virol.2007.07.022 (2007).

6 Toth, K. *et al.* Cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Human gene therapy* **16**, 139-146, doi:10.1089/hum.2005.16.139 (2005).