Journal of Visualized Experiments

Intratracheal administration of dry powder formulation in mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61469R1
Full Title:	Intratracheal administration of dry powder formulation in mice
Section/Category:	JoVE Medicine
Keywords:	insufflator; intratracheal; intubation; orotracheal; powder aerosol; pulmonary delivery
Corresponding Author:	Jenny Lam HONG KONG
Corresponding Author's Institution:	
Corresponding Author E-Mail:	jkwlam@hku.hk
Order of Authors:	Yingshan QIU
	Qiuying LIAO
	Michael Y.T. CHOW
	Jenny Lam
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Hong Kong

TITLE:

Intratracheal Administration of Dry Powder Formulation in Mice

AUTHORS AND AFFILIATIONS:

Yingshan Qiu[1], Qiuying Liao[1], Michael Y.T. Chow[1,2], Jenny K.W. Lam[1]*

[1]Department of Pharmacology and Pharmacy, LKS Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR

[2]Advanced Drug Delivery Group, Sydney Pharmacy School, Faculty of Medicine and Health, The University of Sydney, NSW, Australia

Email addresses of co-authors:

Yingshan Qiu (u3004556@connect.hku.hk)

Qiuying Laio (liaoqy@connect.hku.hk)

Michael Y.T. Chow (yee.chow@sydney.edu.au)

* Corresponding author

Jenny K.W. Lam (jkwlam@hku.hk)

KEYWORDS:

insufflator; intratracheal; intubation; orotracheal; powder aerosol; pulmonary delivery

SUMMARY:

Dry powder formulations for inhalation have great potential in treating respiratory diseases. Before entering human studies, it is necessary to evaluate the efficacy of the dry powder formulation in preclinical studies. A simple and noninvasive method of the administration of dry powder in mice through the intratracheal route is presented.

ABSTRACT:

In the development of inhalable dry powder formulations, it is essential to evaluate their biological activities in preclinical animal models. This paper introduces a noninvasive method of intratracheal delivery of dry powder formulation in mice. A dry powder loading device that consists of a 200 µL gel loading pipette tip connected to 1 mL syringe via a three-way stopcock is presented. A small amount of dry powder (1-2 mg) is loaded into the pipette tip and dispersed by 0.6 mL of air in the syringe. Because pipette tips are disposable and inexpensive, different dry powder formulations can be loaded into different tips in advance. Various formulations can be evaluated in the same animal experiment without needing device cleaning and dose refilling, thereby saving time and eliminating the risk of cross-contamination from residual powder. The extent of powder dispersion can be inspected by the amount of powder remaining in the pipette tip. A protocol of intubation in mouse with a custom-made light source and a guiding cannula is included. Proper intubation is one of the key factors that influences the intratracheal delivery of dry powder formulation to the deep lung region of the mouse.

INTRODUCTION:

The pulmonary route of administration offers various benefits in delivering therapeutics for both local and systemic actions. For the treatment of lung diseases, high local drug concentration can be achieved by pulmonary delivery, thereby reducing the required dose and lowering the incidence of systemic side effects. Moreover, the relatively low enzymatic activities in the lung can reduce premature drug metabolism. The lungs are also efficient for drug absorption for systemic action due to the large and well-perfused surface area, the extremely thin epithelial cell layer and the high blood volume in pulmonary capillaries[1].

Inhaled dry powder formulations have been widely investigated for the prevention and treatment of various diseases such as asthma, chronic obstructive pulmonary disease, diabetes mellitus and pulmonary vaccination[2-4]. Drugs in the solid state are generally more stable than in the liquid form, and dry powder inhalers are more portable and user-friendly than nebulizers[5,6]. In the development of inhaled dry powder formulations, the safety, the pharmacokinetic profile and the therapeutic efficacy need to be evaluated in preclinical animal models following pulmonary administration[7]. Unlike humans who can inhale dry powder actively, pulmonary delivery of dry powder to small animals is challenging. It is necessary to establish an efficient protocol of delivering dry powder to the lungs of animals.

Mice are widely used as animal models because they are economical and they breed well. They are also easy to handle and many disease models are well-established. There are two major approaches to administer dry powder to the lungs of mouse: inhalation and intratracheal administration. For inhalation, the mouse is placed in a whole-body or nose-only chamber where dry powder is aerosolized and the animals breathe in the aerosol without sedation[8,9]. Expensive equipment is required and the drug delivery efficiency is low. While the whole-body chamber may be technically less challenging, the nose-only exposure chamber could minimize exposure of drugs to the body surface. Regardless, it is still difficult to precisely control and determine the dose delivered to the lungs. The dry powder is mainly deposited in the nasopharynx region where mucociliary clearance is prominent[10]. Moreover, mice inside the chamber are under significant stress during the administration process because they are constrained and deprived of food and water supply[11]. For intratracheal administration, it generally refers to the introduction of the substance directly into the trachea. There are two different techniques to achieve this: tracheotomy and orotracheal intubation. The former requires a surgical procedure that makes an incision in the trachea, which is invasive and seldom used for powder administration. Only the second technique is described here. Compared to the inhalation method, intratracheal administration is the more commonly used method for pulmonary delivery in the mouse because of its high delivery efficiency with minimal drug loss[12,13]. It is a simple and fast method to precisely deliver a small amount of powder within a few milligrams to the mouse. Although the mouse is anatomically and physiologically distinct to humans and anesthetization is required during the intubation process, intratracheal administration bypasses the upper respiratory tract and offers a more effective way to assess the biological activities of the dry powder formulation such as the pulmonary absorption, bioavailability and therapeutic effects[14,15].

To administer dry powder intratracheally, the mouse has to be intubated, which could be challenging. In this paper, the fabrication of a custom-made dry powder insufflator and an

intubation device is described. The procedures of intubation and insufflation of dry powder in the lung of the mouse are demonstrated.

PROTOCOL:

The experiments conducted in this study have been approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR), The University of Hong Kong. Dry powder formulations prepared by spray freeze drying (SFD) containing 0.5% of luciferase messenger RNA (mRNA), 5% synthetic peptide $PEG_{12}KL4$ and 94.5% of mannitol (w/w) are used in this study to demonstrate mRNA expression in the lung[16]. The mass median aerodynamic diameter (MMAD) of SFD powder is 2.4 μm. Spray dried (SD) mannitol powder are used to investigate the effect of air volume used in powder dispersion[16]. The MMAD of SD powder is 1.5 μm.

1. Fabrication of dry powder insufflator and loading of dry powder

1.1 Neutralize the static charges of dry powder (in a vial) and the 200 μL non-filter round gel-loading pipette tip. Use an anti-static gun or a balance with deionizing function according to the manufacturer's instruction.

1.2 Prepare a weighing paper with a size of around 4 cm x 4 cm. Fold the paper in half diagonally and then unfold it.

1.3 Weigh 1-2 mg of dry powder on the weighing paper.

1.4 Fill a gel-loading pipette tip with powder through the wider opening of the tip. Tap gently to pack the powder until the powder forms loose agglomerates near the narrow end of the tip (**Figure 1A**). Avoid packing the powder too tightly as it may hamper powder dispersion.

1.5 Connect the powder-loaded tip to a 1 mL syringe through a three-way stopcock (**Figure 1B**). The size of syringe can be changed according to the volume of air used to disperse the powder. Hold the tip and syringe vertically during connection to prevent spillage of powder. If administration is not performed immediately, use parafilm to seal the openings of the tip and store it temporarily under suitable condition until administration.

2. Fabrication of intubation device

2.1 Light source (**Figure 2**)

2.1.1 Prepare a custom-made light source with a light emitting diode (LED) torch and a flexible optical fiber with a diameter of 0.8-1 mm.

2.1.2 Make a centered orifice on the clear lens of the LED torch with a hand drill or a drill bit so that the optical fiber can barely pass through.

2.1.3 Insert the optical fiber through the orifice. Switch on the LED torch to adjust the position and the depth of insertion for maximum brightness at the other end of the optical fiber.

2.1.4 Affix the optical fiber in position with clear epoxy glue.

2.2 Guiding cannula (**Figure 3**)

2.2.1 Take a 1 mL plastic Pasteur pipette (**Figure 3A**) and hold the pipette at both ends.

2.2.2 Use an alcohol lamp (or other heat sources in the laboratory such as a Bunsen burner) to heat the middle of the pipette by placing it at 5-10 cm above the flame (**Figure 3B**). Rotate the pipette to make sure it is heated evenly.

2.2.3 When the plastic becomes soft and deformable, move the pipette away from the flame and stretch the pipette gently.

2.2.4 Cut the stretched pipette in the middle with a pair of scissor into Part A and Part B (**Figure 3C-E**). Use Part A as a fine tip pipette and Part B as a guiding cannula. To increase the chance of successful intubation with the guiding cannula, make a bevel (not too sharp which may increase the risk of injuring the animal) at the end of Part B (**Figure 3F**). When a 200 µL gel-loading pipette tip (for powder loading) is inserted into the guiding cannula, it should protrude the cannula by 1-2 mm.

NOTE: A guiding cannula (Part B) with the appropriate dimension (internal and external diameter) for intubation could have a 21 gauge needle fit inside it while it can also fit inside a 17 gauge needle. Multiple attempts may be needed in stretching the pipettes to achieve the appropriate dimension.

2.2.5 (Optional): Cut a small opening at the wider end of the guiding cannula to make it more flexible so that it is easier to hold the optical fiber (**Figure 3F**). This opening also allows the fitting of a microsprayer for the administration of liquid aerosol.

3. Intubation

3.1 Anaesthetize the mouse (BALB/c, 7-9 weeks) with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection.

3.2 Prepare a platform made of Plexiglass and mount it to a stand with a clamp (**Figure 4A**). Place the anaesthetized mouse on the platform (at around 60° of inclination) in a supine position. The height and angle of inclination of the platform could be adjusted by the position of the clamp on the stand.

3.3 Suspend the mouse by hooking its incisors on a nylon floss (**Figure 4B**). Secure the position of the mouse by a piece of tape or a rubber band.

3.4 Insert the optical fiber into the guiding cannula before intubation with the tip of the fiber level with the opening of the guiding cannula. Turn on the LED torch to illuminate.

3.5 Gently protrude the tongue of the mouse with a pair of forceps to expose its trachea.

3.6 Use the other hand to hold the guiding cannula with optical fiber inside. Insert them through the oral cavity. With the illumination from the optical fiber, the opening of the trachea can be visualized as an orifice between the vocal cords.

3.7 Align the bevel of the guiding cannula towards the midline of the opening (**Figure 5A**). Gently intubate the guiding cannula with optical fiber into the trachea by aiming the finest tip of the cannula at the tracheal opening.

3.8 Upon intubation, swiftly remove the optical fiber and leave the guiding cannula inside the trachea (**Figure 5B**). A normal respiration should be observed.

3.9 Hold the fine tip pipette (Part A) at the opening of the guiding cannula and insufflate a small puff of air (about 0.2 mL) into the lung of the mouse. A slight inflation in the chest of the mouse indicates proper intubation. Remove the fine tip pipette prior to powder administration.

4. Powder administration

4.1 Hold the powder loaded-tip that is connected to the syringe as described in step 1.5. Ensure the airflow between the syringe and the tip is disconnected.

4.2 Pull the syringe plunger backward to withdraw 0.6 mL of air.

NOTE: The volume of air used to disperse the powder is dependent on the properties of the powder and the amount of powder loaded. This is further described in the result section.

4.3 Turn the valve of the three-way stopcock to connect the airflow between the syringe and the powder-loaded tip.

4.4 Insert the powder-loaded tip into the guiding cannula which has already been placed in the trachea of the mouse (**Figure 5C**). Hold the guiding cannula and push the syringe plunger forcefully in one continuous action to disperse the powder as aerosols into the lung.

NOTE: Any forward motion of the device should be minimized to avoid injuring the animal.

4.5 Remove the tip and check if the powder inside the tip has been emptied. If not, repeat step 4.1 to 4.4.

NOTE: If the powder is packed too tightly due to excessive tapping, it might not be dispersed

properly.

4.6 Once the administration is complete, remove the guiding cannula from the trachea.

4.7 Allow the mouse to recover by positioning it horizontally in a supine position with its tongue half protruded to avoid the blockade of the airways.

REPRESENTATIVE RESULTS:

When a dry powder insufflator is used to deliver powder aerosol to the lung of an animal, the volume of air used is critical as it affects the safety as well as the powder dispersion efficiency. To optimize the method, different volumes of air (0.3 mL, 0.6 mL and 1.0 mL) were used to disperse the dry powder (1 mg of spray dried mannitol) and the weight of mice was monitored for 48 hours after administration (**Figure 6**). The use of 0.3 mL and 0.6 mL of air did not cause weight loss of the mice up to 48 h post-administration. Dispersing the powder with 1 mL of air resulted in over 5% of weight loss within 24 h, which was not fully recovered after 48 h. In this protocol, BALB/c mice of 7-9 weeks old were used. Depending on the species, the strain and age of animal, the powder properties (e.g., particle size distribution, cohesiveness and density) and the mass of powder to be administered, the volume of air to be used for efficient powder dispersion and animal tolerance may require optimization by investigators for different animal models.

Dry powder formulation prepared by spray freeze drying (SFD) was delivered to the mice using the method described above. The SFD formulation contained 0.5% of mRNA expressing luciferase protein, 5% of synthetic peptide as delivery vector and 94.5% of mannitol[16]. BALB/c mice were intratracheally administered with 1 mg of SFD powder containing 5 µg of mRNA and the luciferase expression in the lungs was evaluated at 24 h post-administration using in vivo imaging system (IVIS) (**Figure 7**). The SFD powder were dispersed in the deep lung and luciferase expression was observed. As a comparison, the SFD powder were reconstituted in water (to a final volume of 75 µL) and administered to mice as liquid with the same intubation procedure but a microsprayer was used instead to generate liquid aerosol[16]. The luciferase expression of the reconstituted formulation was significantly higher than the dry powder formulation, which could be due to the powder dissolution issue or different pharmacokinetic profile between powder and liquid form. The histological characteristics of the lungs treated with mRNA dry powder aerosol were compared with untreated control and lipopolysaccharide (LPS) treated groups (**Figure 8**). The lungs without any treatment illustrated a healthy presentation while the lung treated with 10 µg of LPS intratracheally showed irregular distribution of air space and inflammatory cell infiltration into the interstitial and alveolar spaces. The lungs treated with SFD powder did not show any signs of inflammation.

TABLE AND FIGURE LEGENDS:

Figure 1: Custom-made dry powder insufflator. (**A**) Powder is packed near the narrow end of the tip. (**B**) A gel-loading pipette tip is connected to a 1 mL syringe via a three-way stopcock. The figure is adapted from Liao et al. [21].

Figure 2: Custom-made light source for intubation. A flexible optical fiber is connected to a LED torch by creating a small hole on the lens.

Figure 3: Guiding cannula. (**A**) A 1 mL plastic Pasteur pipette is used to make a guiding cannula. (**B**) The pipette is softened by heating. (**C**) The heated pipette is stretched and cut. (**D**) Part A of the pipette is used as fine-tip pipette. (**E&F**) Part B of the pipette is used as a guiding cannula. A bevel is created to facilitate intubation procedure. A small opening (optional) can be made to increase the flexibility of the cannula.

Figure 4: Intubation platform. (**A**) The platform for intubation consists of a Plexiglass plate which is mounted to a stand. (**B**) An anaesthetized mouse is placed on the platform in a supine position, suspended by hooking its incisors with a nylon floss.

Figure 5: Schematic diagram illustrating the intubation procedure. (**A**) The bevel of the guiding cannula is aligned with the midline of the tracheal opening. (**B**) The guiding cannula is inserted into the trachea and ready for powder administration. (**C**) The powder-loaded tip (connected to the syringe through a three-way stopcock) is inserted into the guiding cannula which has already been placed in the trachea of the mouse.

Figure 6: Intratracheal administration of dry powder with different volume of air. BALB/c mice were administered intratracheally with spray dried (SD) mannitol powder dispersed by 0.3 mL, 0.6 mL and 1.0 mL of air. Body weight of the mice was monitored before administration and at 18 h, 24 h and 48 h post-administration. The data was presented as mean value of percentage of weight change (n=2).

Figure 7: Intratracheal administration of mRNA formulation as dry powder and reconstituted liquid aerosol. BALB/c mice were administered intratracheally with spray freeze dried (SFD) 0.5% mRNA (luciferase) formulation as powder aerosol (1 mg) using custom-made dry powder insufflator or reconstituted liquid aerosol (1 mg in 75 μL PBS) using microsprayer. Each mouse received a dose of 5 μg of mRNA. PBS (75 μL) was used as control. At 24 h post-administration (A) the lungs were isolated for bioluminescence imaging; (B) luciferase protein expression of the lung tissues were measured. The data was expressed as the mean value of relative light unit (RLU) per mg of protein, analyzed by one-way ANOVA followed by Tukey's post-hoc test, $^{***}p < 0.001$ (n=4). The figure is adapted from Qiu et al. [16].

Figure 8: Histology of the lungs of mice following intratracheal administration of mRNA dry powder formulation. (**A**) untreated control; mice were intratracheally administered with (**B**) LPS (10 mg in 25 μL PBS), and (**C**) spray freeze dried mRNA powder (1 mg). Slides were viewed using an upright microscope at 20x magnification (scale bar = 100 mm). The figure is adapted from Qiu et al. [16].

DISCUSSION:

In this paper, custom-made devices for dry powder insufflation and intratracheal intubation are presented. In the powder loading step, dry powder are loaded into a 200 μL gel-loading pipette

tip. It is important to gently tap the tip to allow the loose packing of powder at the narrow end of the tip. However, if the powder are packed too tightly, they will get stuck in the tip and cannot be properly dispersed. It is recommended to neutralize the static charges of the powder and the pipette tip in order to facilitate powder loading, particularly for powder with low density and in low relative humidity. The guiding cannula is a critical component of the device. It is used to facilitate the intubation of powder-loaded pipette tip into the trachea of the mouse. The diameter of the guiding cannula should not be too wide; otherwise it will be difficult to insert it into the trachea and may injure the mouse. The diameter of the guiding cannula should be just wide enough to fit the optical fiber and the powder-loaded pipette tip, and the pipette tip should protrude the guiding cannula by approximately 1-2 mm.

The ability to visualize the opening of the trachea is crucial in the intubation process, allowing the guiding cannula to be correctly inserted. The tracheal opening consists of white arytenoid cartilage with regular opening and closing motion at the back of the throat. With the fiber optic illumination, the opening of trachea could be easily visualized. By puffing a tiny volume of air through the fine tip plastic pipette, an inflation at the chest indicates a proper intubation. If inflation at the chest is not observed or resistance is felt during insertion, retract the guiding cannula swiftly and repeat the steps again.

There was a widely used commercially available dry powder insufflator[12,17,18] (**Table of Materials**; this device is now discontinued). The dry powder is loaded into the sample chamber of the device and dispersed by air from a 3 mL plastic air syringe or an air pump. To measure the emitted dose, the device has to be weighed before and after powder administration, which leads to inaccuracy considering the dose of powder is usually very small (relative to the mass of the device). Compared to the commercial insufflator, the biggest advantage of the custom-made device is that the success of powder dispersion could be observed by the absence of powder in the transparent gel-loading pipette tips. Since the pipette tip is light, it can also be weighed accurately before and after administration to measure the emitted dose. The pipette tip is inserted into the guiding cannula rather than being exposed to the trachea of the animal. There is minimal risk of contaminating the tip with the moisture or secretion in the trachea (which may affect the accuracy of emitted dose measurement). As the pipette tips are disposable and inexpensive, different dry powder formulations can be loaded into different tips in advance. Various formulations can be evaluated in the same animal experiment without the need of device cleaning and dose refilling, thereby saving time and eliminating the risk of cross-contamination from residual powder. Moreover, the powder dispersion pattern generated by the commercial insufflator varied among different formulations. A number of studies reported that dry powder dispersed by the commercial insufflator were easily agglomerated and failed to reach the deep lung upon administration[19,20]. In contrast, other formulations dispersed by devices similar to ours are reported to have a high lung deposition[15,21,22].

There are other similar custom-made devices reported in the literature for the administration of powder aerosol to the lung of animal. For instance, Chaurasiya et al. described the use of a cannula tube for intubation as well as powder loading, with a syringe connected to the cannula tube after intubation for powder dispersion[23]. While their approach uses standardized

equipment and material (e.g., otoscope, cannula and syringe) with less customization, the method here offers some distinct advantages. Firstly, it allows confirmation of correct intubation before drug administration. This step is particularly helpful for less experienced user. Secondly, the guiding cannula can act as a protecting shield to prevent any secretion or moisture in the trachea from contaminating the gel-loading pipette tip, allowing a more accurate emitted dose measurement by weighing. Lastly, the more flexible guiding cannula together with the optical fiber may enable easier intubation.

In summary, a custom-made dry powder insufflator which is inexpensive, disposable, reproducible and efficient in dispersing small amount of powder precisely is introduced in this paper. The intubation process mentioned is noninvasive, quick and could deliver powder formulations to the mice safely and accurately. It can also be adopted to administer liquid formulation for pulmonary delivery.

ACKNOWLEDGMENTS:

The authors would like to thank Mr. Ray Lee, Mr. HC Leung and Mr. Wallace So for their kind assistance in making the light source and powder insufflator; and the Faculty Core Facility for the assistance in animal imaging. The work was supported by the Research Grant Council, Hong Kong (17300319).

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1 Newman, S. P. Drug delivery to the lungs: challenges and opportunities. *Therapeutic Delivery.* **8** (8), 647-661 (2017).

2 Setter, S. M. et al. Inhaled dry powder insulin for the treatment of diabetes mellitus. *Clinical Therapeutics.* **29** (5), 795-813 (2007).

3 Muralidharan, P., Hayes, D., Jr., Mansour, H. M. Dry powder inhalers in COPD, lung inflammation and pulmonary infections. *Expert Opinion on Drug Delivery.* **12** (6), 947-962 (2015).

4 de Boer, A. H. et al. Dry powder inhalation: past, present and future. *Expert Opinion on Drug Delivery.* **14** (4), 499-512 (2017).

5 Das, S., Tucker, I., Stewart, P. Inhaled dry powder formulations for treating tuberculosis. *Current Drug Delivery.* **12** (1), 26-39 (2015).

6 Okamoto, H. et al. Stability of chitosan-pDNA complex powder prepared by supercritical carbon dioxide process. *International Journal of Pharmaceutics.* **290** (1-2), 73-81 (2005).

7 He, J. et al. Evaluation of inhaled recombinant human insulin dry powders: pharmacokinetics, pharmacodynamics and 14-day inhalation. *Journal of Pharmacy and Pharmacology.* **71** (2), 176-184 (2019).

8 Durham, P. G., Young, E. F., Braunstein, M. S., Welch, J. T., Hickey, A. J. A dry powder combination of pyrazinoic acid and its n-propyl ester for aerosol administration to animals. *International Journal of Pharmaceutics.* **514** (2), 384-391 (2016).

9 Phillips, J. E., Zhang, X., Johnston, J. A. Dry powder and nebulized aerosol inhalation of pharmaceuticals delivered to mice using a nose-only exposure system. *JoVE (Journal of Visualized*

Experiments). (122), e55454 (2017).
10 Nahar, K. et al. In vitro, in vivo and ex vivo models for studying particle deposition and drug absorption of inhaled pharmaceuticals. *European Journal of Pharmaceutical Sciences.* **49** (5), 805-818 (2013).
11 Price, D. N., Muttil, P. Delivery of Therapeutics to the Lung. *Methods in Molecular Biology.* **1809** 415-429 (2018).
12 Chang, R. Y. K. et al. Proof-of-Principle Study in a Murine Lung Infection Model of Antipseudomonal Activity of Phage PEV20 in a Dry-Powder Formulation. *Antimicrobial Agents and Chemotherapy.* **62** (2) (2018).
13 Ito, T., Okuda, T., Takayama, R., Okamoto, H. Establishment of an Evaluation Method for Gene Silencing by Serial Pulmonary Administration of siRNA and pDNA Powders: Naked siRNA Inhalation Powder Suppresses Luciferase Gene Expression in the Lung. *Journal of pharmaceutical sciences.* **108** (8), 2661-2667 (2019).
14 Patil, J. S., Sarasija, S. Pulmonary drug delivery strategies: A concise, systematic review. *Lung India.* **29** (1), 44-49 (2012).
15 Ihara, D. et al. Histological Quantification of Gene Silencing by Intratracheal Administration of Dry Powdered Small-Interfering RNA/Chitosan Complexes in the Murine Lung. *Pharmaceutical Research.* **32** (12), 3877-3885 (2015).
16 Qiu, Y. et al. Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. *Journal of Controlled Release.* **314** 102-115 (2019).
17 Pfeifer, C. et al. Dry powder aerosols of polyethylenimine (PEI)-based gene vectors mediate efficient gene delivery to the lung. *Journal of Controlled Release.* **154** (1), 69-76 (2011).
18 Kim, I. et al. Doxorubicin-loaded highly porous large PLGA microparticles as a sustained-release inhalation system for the treatment of metastatic lung cancer. *Biomaterials.* **33** (22), 5574-5583 (2012).
19 Tonnis, W. F. et al. A novel aerosol generator for homogenous distribution of powder over the lungs after pulmonary administration to small laboratory animals. *European Journal of Pharmaceutics and Biopharmaceutics.* **88** (3), 1056-1063 (2014).
20 Hoppentocht, M., Hoste, C., Hagedoorn, P., Frijlink, H. W., de Boer, A. H. In vitro evaluation of the DP-4M PennCentury insufflator. *European Journal of Pharmaceutics and Biopharmaceutics.* **88** (1), 153-159 (2014).
21 Liao, Q. et al. Porous and highly dispersible voriconazole dry powders produced by spray freeze drying for pulmonary delivery with efficient lung deposition. *International Journal of Pharmaceutics.* **560** 144-154 (2019).
22 Ito, T., Okuda, T., Takashima, Y., Okamoto, H. Naked pDNA Inhalation Powder Composed of Hyaluronic Acid Exhibits High Gene Expression in the Lungs. *Molecular Pharmaceutics.* **16** (2), 489-497 (2019).
23 Chaurasiya, B., Zhou, M., Tu, J., Sun, C. Design and validation of a simple device for insufflation of dry powders in a mice model. *European Journal of Pharmaceutical Sciences.* **123** 495-501 (2018).

Figure 1

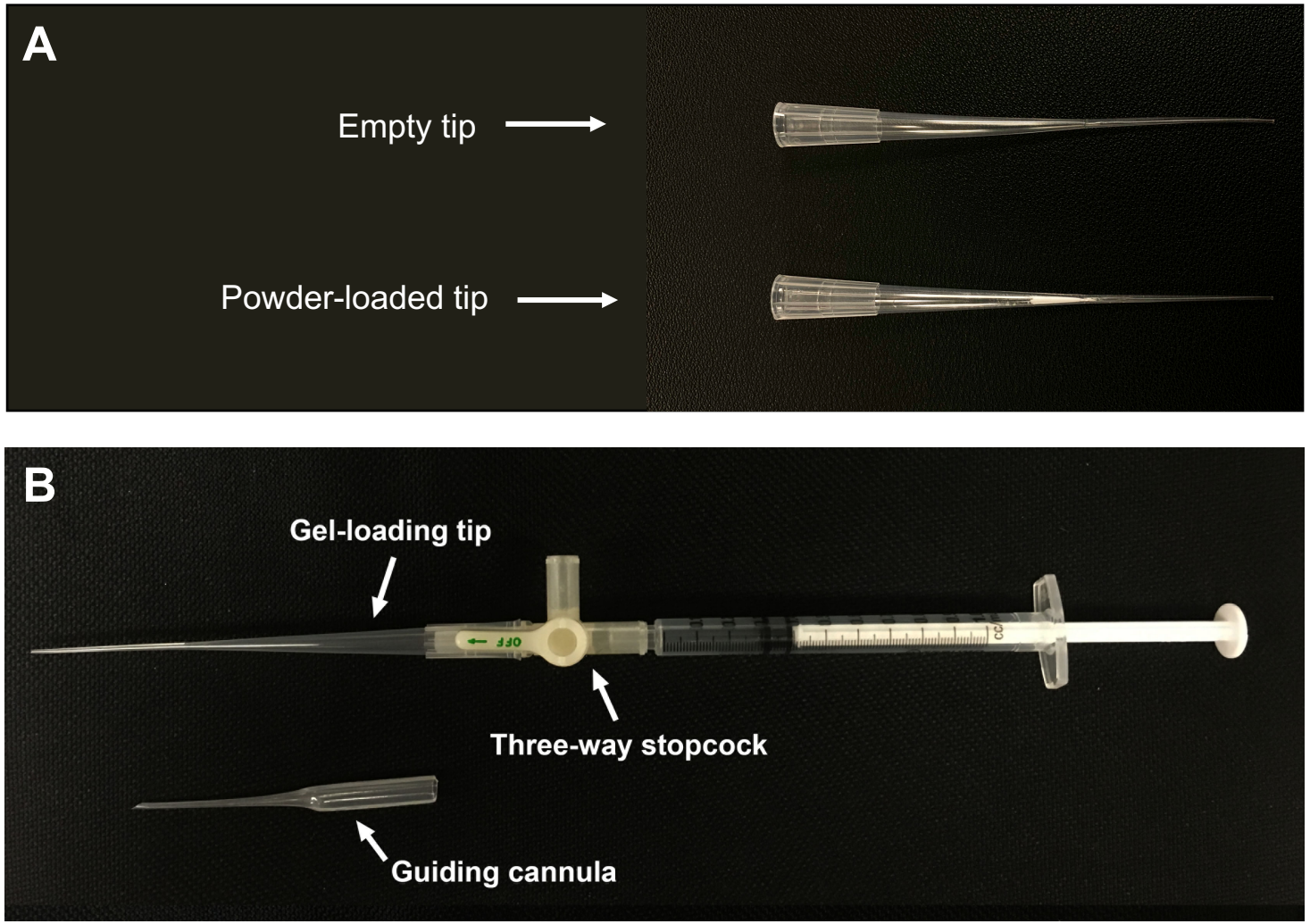


Figure 2

Click here to access/download;Figure;Figure 2.pdf

Figure 3

Click here to access/download;Figure;Figure 3.pdf

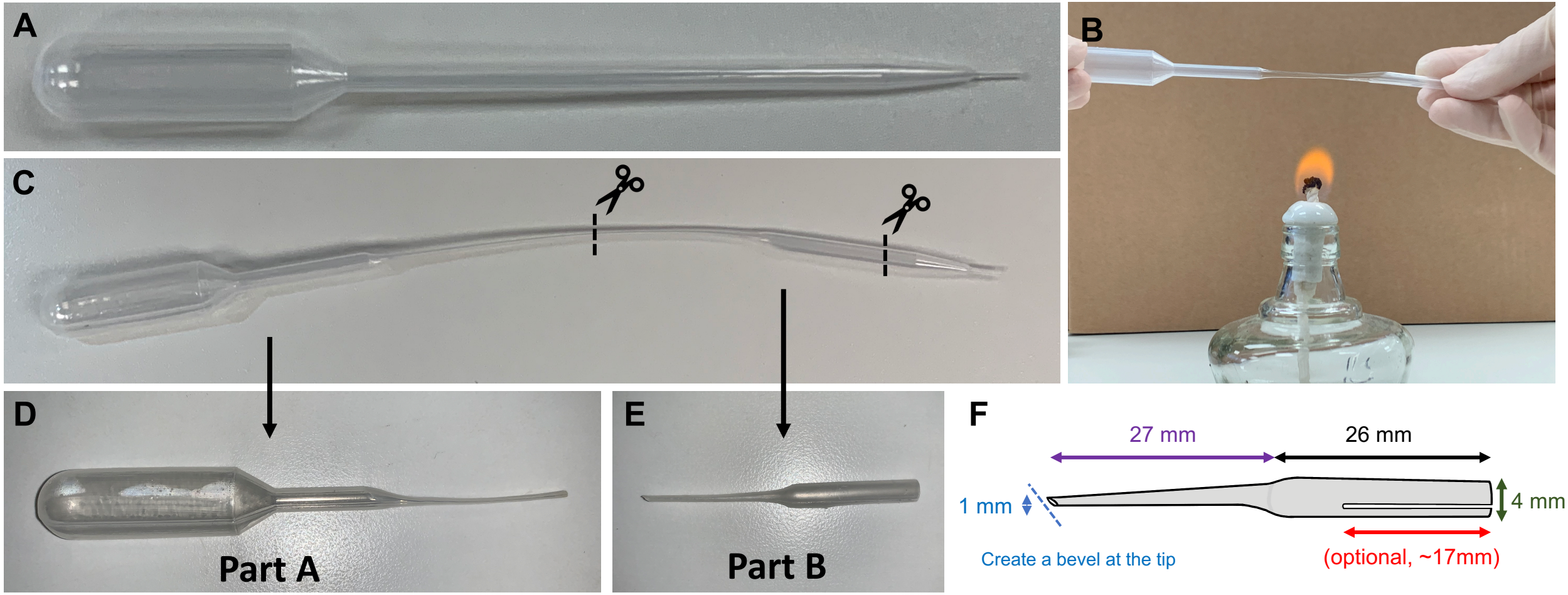


Figure 4

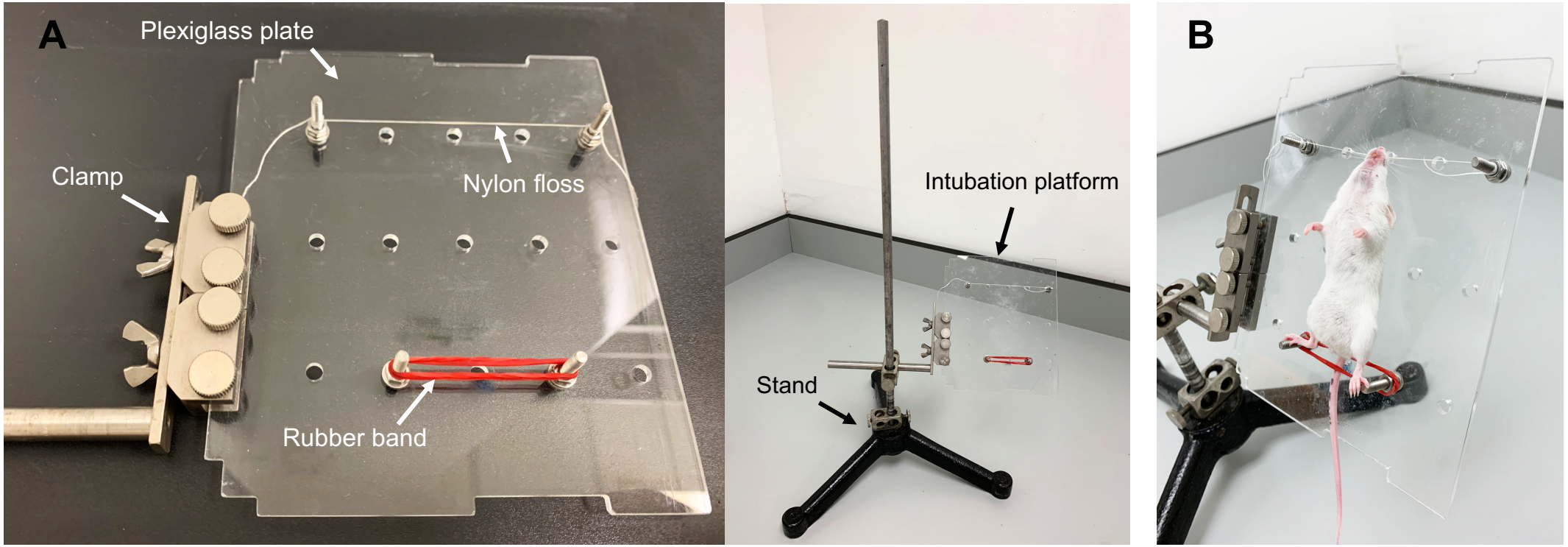


Figure 5

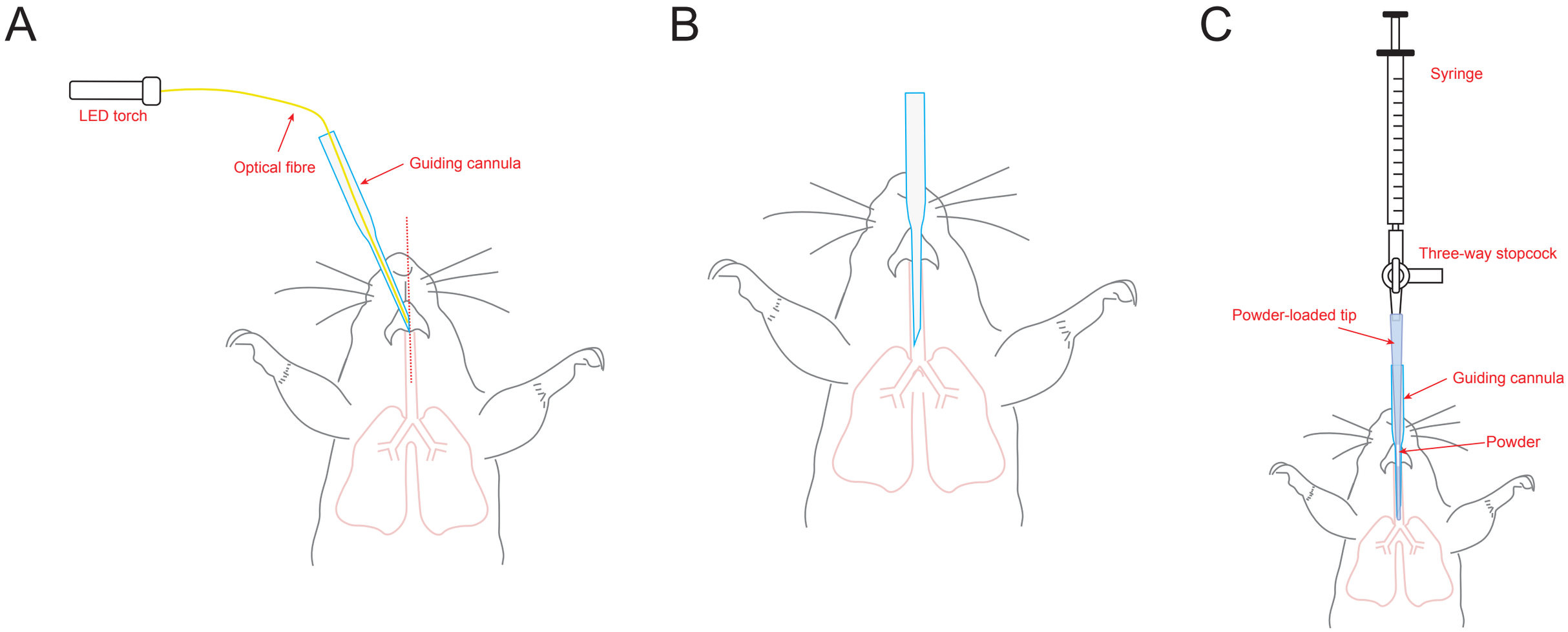


Figure 6

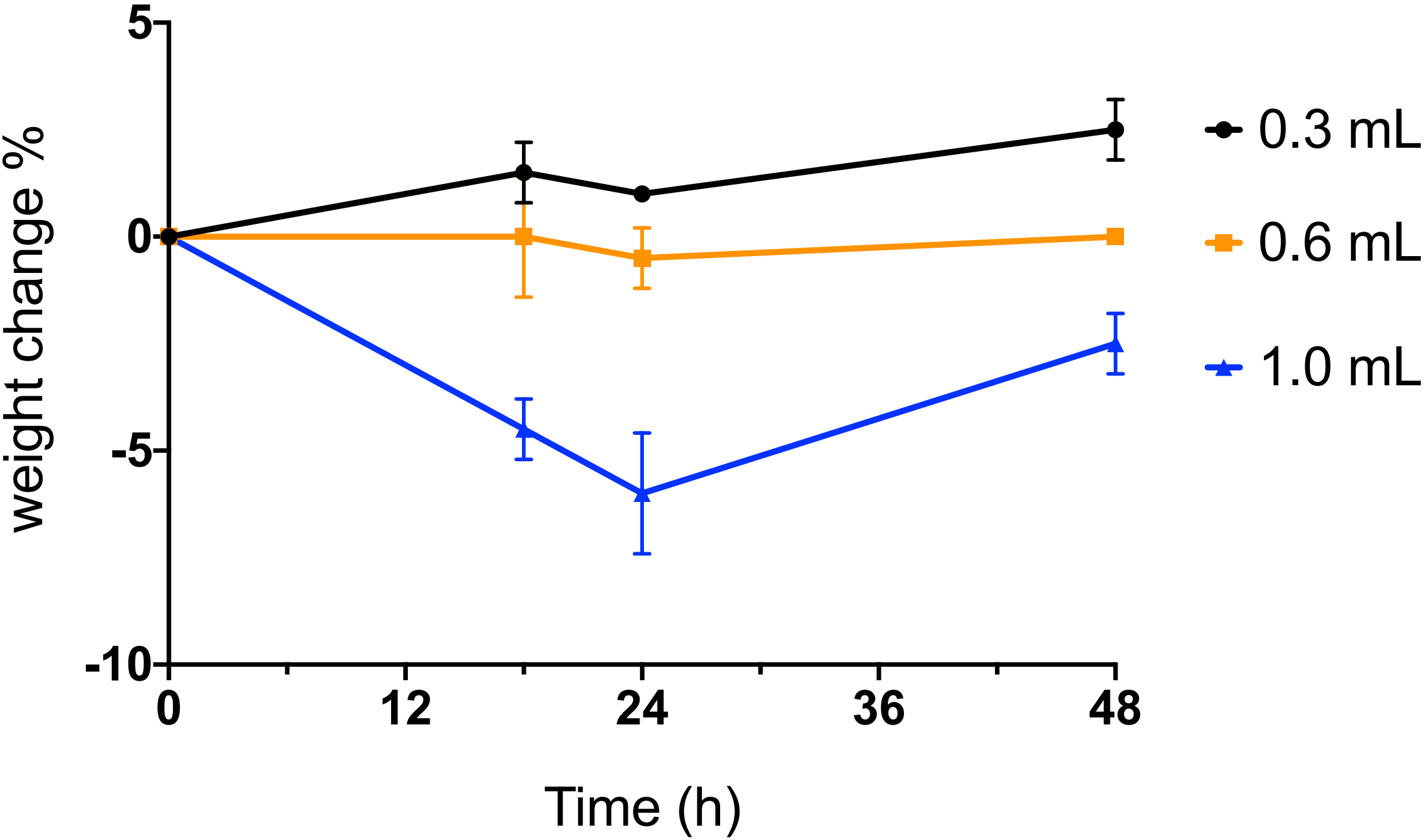


Figure 7

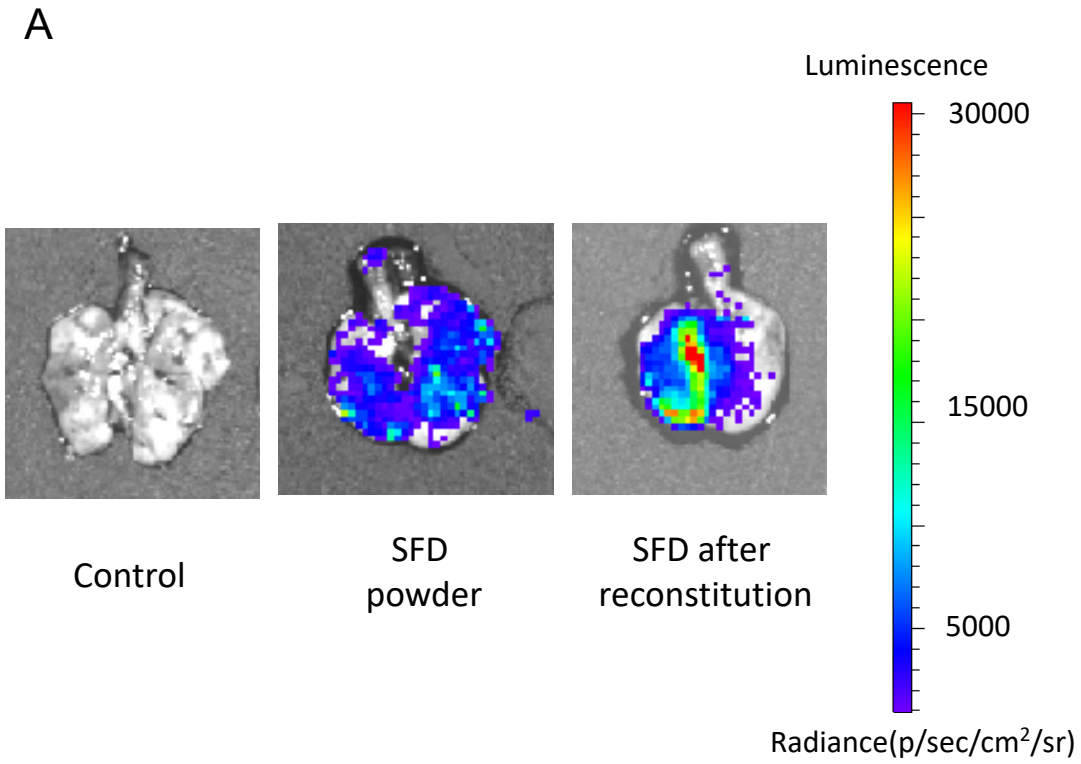
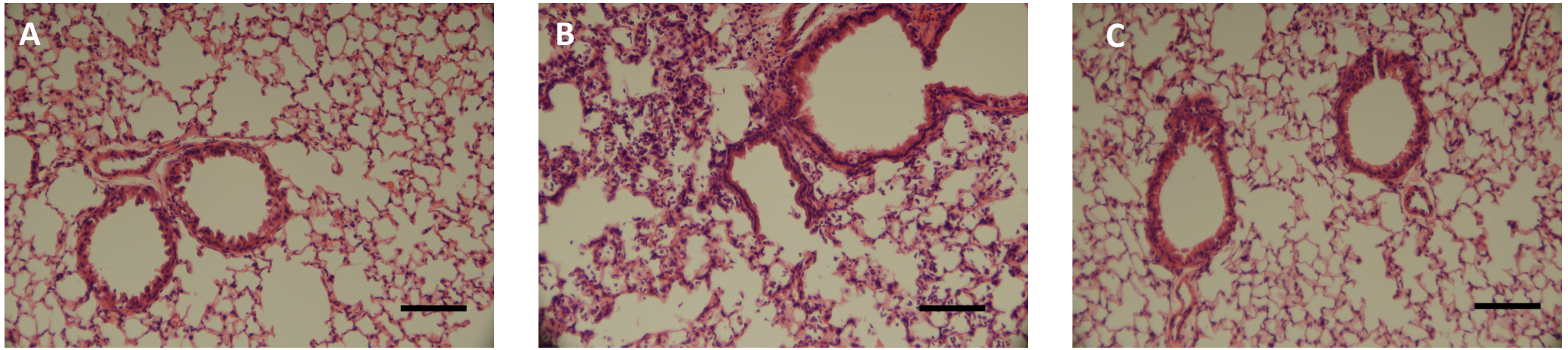


Figure 8



Name of Material/ Equipment	Company	Catalog Number
BALB/c mouse		
CleanCap Firefly Luciferase mRNA	TriLink Biotechnology	L-7602
Dry Powder Insufflator	PennCentury	Model DP-4M
Ketamine 10%	Alfasan International B.V.	NA
Light emitting diode (LED) torch	Unilite Internation	PS-K1
Mannitol (Pearlitol 160C)	Roquette	450001
Non-filter round gel loading pipette tip (200 µL)	Labcon	1034-800-000
Nylon floss	Reach	30017050
One milliliter syringe without needle	Terumo	SS-01T
Optical fibre	Fibre Data	OMPF1000
$PEG_{12}KL4$ peptide	EZ Biolab	
Plastic Pasteur fine tip pipette	Alpha Labotatories	LW4061
Three-way stopcock	Braun	D201
Xylazine 2%	Alfasan International B.V.	NA
Zerostat 3 anti-static gun	MILTY	5036694022153

Comments/Description
Female; 7-9 weeks old; Body weight 20-25 g

(PEG12)-KLLLLKLLLLKLLLLKLLLLK-NH2

HKU Med LKS Faculty of Medicine
Department of Pharmacology & Pharmacy
香港大學藥理及藥劑學系

林嘉穎副教授
藥理及藥劑學系

Dr Jenny K W Lam
Associate Professor
email: jkwlam@hku.hk

17 June 2020

Dear Editors of *Journal of Visualized Experiments*,

Thank you for considering our manuscript titled '*Intratracheal administration of dry powder formulation in mice*'. We appreciate the valuable comments from the editor and reviewers. We have improved the manuscript in response to the suggestions. We highlighted the changes in the manuscript. Our responses to the comments are shown below:

Response to Editorial Comments

1. The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.

   Thanks for the comments, we have revised the manuscript to improve the language of the manuscript.

2. Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
   1) 1.1: Mention all relevant specifications about the powder. What is the chemical composition? What is the particle size?
   2) 3.1: mention animal strain

   Thanks for the comments, we have revised the manuscript to include more specific details.

3. Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):
   1) modifications and troubleshooting,
   2) limitations of the technique,
   3) significance with respect to existing methods,
   4) future applications and
   5) critical steps within the protocol.

   Thanks for the comments, we have revised the discussion of manuscript to address the comments above.

4. References: Please spell out journal names.

   We have made the changes accordingly.

5. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company

HKU Med LKS Faculty of Medicine
Department of Pharmacology & Pharmacy
香港大學藥理及藥劑學系

林嘉穎副教授
藥理及藥劑學系

Dr Jenny K W Lam
Associate Professor
email: jkwlam@hku.hk

brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MicroSprayer®, (PennCentury™)

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have made the changes accordingly.

6. Table of Materials:
   1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as powder, animal strain.
   2) Sort items in alphabetical order.

We have made the changes accordingly.

7. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. 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]."

The re-print permission is uploaded in the supplementary files section as suggested, and the figure is cited accordingly.

Response to Reviewer 1

1. The authors stated in the introduction that "Dry powder formulation could be delivered to the deep lung region of the mouse intratracheally by proper intubation". I do not think this viewpoint is correct. intubation is only one of the factors that may influence the delivery of dry powders into the deep lung. Pls revise.

   Thanks for the comment. We have now revised the manuscript to clarify that intubation is only one of the factors that influence the delivery of dry powders into the deep lung.

2. As the powder-loaded tip is upstanding before insufflation, I am curious if the powders could be still kept in the tip without flowing out.

   For fine powders engineered to be inhalable in human, *i.e.* particles with aerodynamic diameters in the range of 1 – 5 µm, they are generally sufficiently cohesive (particularly after gentle tapping) to

HKU Med LKS Faculty of Medicine
Department of Pharmacology & Pharmacy
香港大學藥理及藥劑學系

林嘉穎副教授
藥理及藥劑學系

Dr Jenny K W Lam
Associate Professor
email: jkwlam@hku.hk

remain inside the tip until a pressure of dispersion has been applied. In our experience, the powder could be kept in the tip without flowing out.

3. The authors create a bevel at the tip of the guiding cannula. However, this dramatically increases the risk to damage the trachea during intubation.

   We appreciate the comment. There may be an increased risk (moderate risk at most) of damaging the trachea during intubation. Therefore, we recommend not to create a sharp angle at the tip, but a relatively blunt bevel to reduce the risk of injury while increasing the chance of successful intubation. We have added this information in the revised manuscript.

4. Pls perform more experiments to validate the device.

   We have published papers to demonstrate the administration of powder formulation using the device (Liao et al 2019; Qiu et al 2019;). Similar device has also been reported by other groups (Ihara et al 2015; Ito et al 2019; Miwata et al 2018). Please refer to those papers for details of experiments

   - Ihara, D. et al. Histological Quantification of Gene Silencing by Intratracheal Administration of Dry Powdered Small-Interfering RNA/Chitosan Complexes in the Murine Lung. Pharmaceutical Research. 32 (12), 3877-3885, (2015).
   - Ito, T., Okuda, T., Takashima, Y. & Okamoto, H. Naked pDNA Inhalation Powder Composed of Hyaluronic Acid Exhibits High Gene Expression in the Lungs. Molecular Pharmaceutics. 16 (2), 489-497, (2019).
   - Liao, Q. et al. Porous and highly dispersible voriconazole dry powders produced by spray freeze drying for pulmonary delivery with efficient lung deposition. International Journal of Pharmaceutics. 560 144-154, (2019).
   - Miwata, K. et al. Intratracheal Administration of siRNA Dry Powder Targeting Vascular Endothelial Growth Factor Inhibits Lung Tumor Growth in Mice. Molecular Therapy - Nucleic Acids. 12 698-706, (2018).
   - Qiu, Y. et al. Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. Journal of Controlled Release. 314 102-115, (2019).

5. Please mention the reconstituted SFD powder was delivered by using PennCentury.

   We have included this information in the revised manuscript.

6. Birendra et al. recently reported a similar device in EJPS (https://doi.org/10.1016/j.ejps.2018.08.010), pls cite and comment.

   Thanks for the comment. We have cited this article, and made a brief comparison between the two devices. Birendra et al. described the method that uses a 20 G cannula tube for intubation as well as powder loading. A syringe is connected to the cannula tube after intubation for powder dispersion. All the materials used (e.g. otoscope, cannula and syringe) are standardized. In our method, intubation is performed with a separate guiding canula followed by powder administration through the syringe connected gel-loading tip. While the two approaches appear to be similar, ours has

HKU Med LKS Faculty of Medicine
Department of Pharmacology & Pharmacy
香港大學藥理及藥劑學系

林嘉穎副教授
藥理及藥劑學系

Dr Jenny K W Lam
Associate Professor
email: jkwlam@hku.hk

offered several distinct advantages. Firstly, it allows confirmation of correct intubation (to the trachea but not the oesophagus) before drug administration. This step is particularly helpful for less experienced user. Secondly, the guiding cannula can act as a protecting shield to prevent any secretion or moisture in the trachea from contaminating the gel-loading tip, allowing a more accurate emitted dose measurement by weighing. Lastly, the more flexible guiding cannula together with the optical fibre may enable easier intubation. We have included these information in the revised manuscript.

Response to Reviewer 2

1. Is the gel-loading tip length adjustable? How the operator can determine the right length?

   The length of the gel-loading tip is not adjusted and is used as it is. This would be more user-friendly as the powder can be loaded into the tip without the need for length adjustment each time. The depth of intubation is determined by the dimension of the guiding cannula, which also serves as a holder to the gel-loading tip. When the gel-loading tip was fully inserted into the guiding cannula, the end of the tip should be only slightly protruded.

2. It seems from Figure 6 that the volume of air is affecting the animal body weight, how many ml of air do the author suggest to administer to mice or rat? Is 1 ml the maximum volume suggested?

   The volume of air used to disperse the powder depends on a number of factors: (i) the species and strain of animals; (ii) the age of animals; (iii) the properties of the powder (e.g. particle size distribution, cohesiveness and density); and (iv) the mass of powder to be administered. Operators should first determine the minimal volume required to disperse the powder in vitro, and then test for tolerance in animal. We have included these information in the revised manuscript.

3. In order to increase the volume of air, is the syringe capacity changeable ?

   Yes. The size of syringe can be changed for different volume of air used to disperse the powder. We have included this information in the revised manuscript.

4. Could be the system adaptable to administer liquid pulmonary formulation?

   Yes, the incubation procedure can be adopted to administer liquid formulation for pulmonary delivery. We have included this information in the revised manuscript.

5. Can you weight the gel loading tip before and after the administration to measure the emitted powder dose?

   Yes. Users can weigh the gel-loading tip before and after the administration to measure the emitted powder dose with a highly sensitive analytical balance. Since the gel loading tip is inserted into the guiding cannula rather than being directly exposed to the trachea of the animal, there is a minimal risk of contaminating the gel loading tip with moisture/ secretion in the trachea. We have included this information in the revised manuscript.

HKU Med LKS Faculty of Medicine
Department of Pharmacology & Pharmacy
香港大學藥理及藥劑學系

林嘉穎副教授
藥理及藥劑學系

Dr Jenny K W Lam
Associate Professor
email: jkwlam@hku.hk

Response to Reviewer 3

1. Numerous English language errors that can be easily remedied.

   We have improved the language of the revised manuscript.

2. In the abstract and discussion, the authors mention that the dry powder formulations can be "delivered to multiple mice at the same time". This need to be changed, as it suggests that a single person can deliver a formulation to many mice all at once (as could occur with a nose-only chamber that had multiple chambers for multiple mice), which is simply not the case. The sentence must be rewritten.

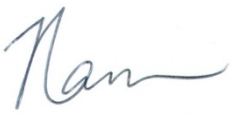
   We appreciate the comment. The sentence is now rephrased in the revised manuscript to read 'Because the pipette tips are disposable and inexpensive, different dry powder formulations can be loaded into different tips in advance. Various formulations can be evaluated in the same animal experiment without the need of device cleaning and dose refilling, thereby saving time and eliminating the risk of cross-contamination from residual powder.'

3. Figure 7 - I'm bothered by the lack of a control in both the lung images and the quantified data. A non-mRNA (or naked mRNA) containing powder delivered to mice and imaged should be included

   We appreciate the comment. We have included a control group in Figure 7 of the revised manuscript.

We hope the changes will make the manuscript suitable for publication in the *Journal of Visualized Experiments*.

Yours sincerely,



Jenny K W Lam

Corresponding author: Jenny K W Lam, Department of Pharmacology & Pharmacy, LKS Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong; jkwlam@hku.hk; phone: (852) 3917 9599; fax: (852) 2817 0859

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 21, 2020

This Agreement between The University of Hong Kong -- Qiuying Liao ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4833700892222
License date	May 21, 2020
Licensed Content Publisher	Elsevier
Licensed Content Publication	International Journal of Pharmaceutics
Licensed Content Title	Porous and highly dispersible voriconazole dry powders produced by spray freeze drying for pulmonary delivery with efficient lung deposition
Licensed Content Author	Qiuying Liao,Long Yip,Michael Y.T. Chow,Shing Fung Chow,Hak-Kim Chan,Philip C.L. Kwok,Jenny K.W. Lam
Licensed Content Date	Apr 5, 2019
Licensed Content Volume	560
Licensed Content Issue	n/a
Licensed Content Pages	11
Start Page	144
End Page	154
Type of Use	reuse in a journal/magazine

Requestor type	academic/educational institute
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of new article	Intratracheal administration of dry powder formulation in mice
Lead author	Jenny Lam
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp.
Expected publication date	Jun 2020
Portions	Supplementary Figure S1
Requestor Location	The University of Hong Kong L2-02, 2/F, Lab Block 21 Sassoon Road Hong Kong, 518000 Hong Kong Attn: The University of Hong Kong
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant

DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 21, 2020

This Agreement between The University of Hong Kong -- Qiuying Liao ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4833701203241
License date	May 21, 2020
Licensed Content Publisher	Elsevier
Licensed Content Publication	Journal of Controlled Release
Licensed Content Title	Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide
Licensed Content Author	Yingshan Qiu,Rico C.H. Man,Qiuying Liao,Keshia L.K. Kung,Michael Y.T. Chow,Jenny K.W. Lam
Licensed Content Date	Nov 28, 2019
Licensed Content Volume	314
Licensed Content Issue	n/a
Licensed Content Pages	14
Start Page	102
End Page	115
Type of Use	reuse in a journal/magazine

Requestor type	academic/educational institute
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of new article	Intratracheal administration of dry powder formulation in mice
Lead author	Jenny Lam
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp.
Expected publication date	Jun 2020
Portions	Figure 10, Figure 12
Requestor Location	The University of Hong Kong L2-02, 2/F, Lab Block 21 Sassoon Road Hong Kong, 518000 Hong Kong Attn: The University of Hong Kong
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant

DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.