Standard Operating Procedure (SOP) for
Bead Model of *Aspergillus fumigatus* Colonization
NIH/NIAID Task Order A05

1. Purpose:
This Standard Operating Procedure (SOP) will provide information necessary for the colonization of mice airways with *Aspergillus fumigatus* hyphae

2. Scope:
This SOP will provide sufficient information to infect mice with *A. fumigatus* conidia embedded inside agar beads. The purpose of this procedure is to induce the colonization of mouse airways with *A. fumigatus* without the use of immunosuppressive therapy. This SOP introduces the process of encapsulating *A. fumigatus* conidia inside agar beads and describes the infection procedure and the monitoring of infected mice.

3. Definitions:
For the purposes of this SOP, "infect" will mean to introduce into the animal a precise, quantified concentration of viable *Aspergillus fumigatus* conidia encapuslated within Yeast-Peptone-Dextrose agar beads.

4. Responsibilities:
This SOP shall be utilized by employees of Research assistant status or higher without additional training. Research technicians may perform this work upon receipt of training.

5. Equipment:
- Drugs
  - Isoflurane (Fresenius Kabi, CDMV)
- Mice – female C57BL/6, ~20g; Charles River, strain #027
  - Acceptable equivalent: male C57BL/6, Charles River
- 70% ethanol
- Yeast-Peptone-Dextrose media supplemented with 3% agar
- Paraffin oil (Fisher cat# F 0122-4) supplied as 4 liter jug
- Phosphate buffered saline (PBS)
- Phosphate buffered saline + 0.1% Tween-80 (PBS-T)
- Pasteur pipettes (glass)
- 50 ml Oak Ridge high-speed centrifuge tubes
- 15 ml Polystyrene tubes
- 50 ml conical tubes
- 500 ml flask (sterile)
- Metal mesh (Bellco Glass Inc. cat# 1985-00050)
- Homogenizer (Kinematica GMBH, Brinkmann Instruments Co.)
- Curved 26-gauge needles
- 1 ml syringes
- Surgical microscope
- Sterile paper clip

- Gauze swab cloths

6. Procedure:

- Preparation of inoculum

The inoculum should be prepared according to the procedures outlined in the following SOP available on the IAAM website (http://www.sacmm.org/sop.html): Standard Operating Procedures for Preparation of Aspergillus fumigatus Test Strains for Inhalation Pulmonary Aspergillosis Animal Models

- Preparation of agar beads
    - Warm 150 ml sterile paraffin oil to 52˚C in a sterile 500 ml flask.
    - Warm 10ml of Yeast-Peptone-Dextrose media supplemented with 3% agar (= 2x YPD agar) solution to 52˚C. Mix 5 ml of freshly harvested *A. fumigatus* conidial suspension in PBS/0.1% Tween 80 (1x10^9 conidia/ml) with 5 ml of 2x YPD agar as follows: First, fill a prewarmed 10 ml disposable pipette with 5ml of the 2x YPD agar. Next, aspirate 5ml of the conidial suspension into the same pipette for a total volume of 10ml. The two solutions will mix during aspiration.
    - Immediately, pipette the mixture containing conidia and YPD agar forcefully (maximum speed on PipetteAid) into the middle of a 500 ml flask containing 150 ml of pre-warmed (52˚C) sterile paraffin oil. The oil should be stirred rapidly with magnetic bar during the pipetting and for 6 min further, at room temperature.
    - Cool the mixture by piling crushed ice around the flask (surround flask with aluminium foil boat before starting the experiment) for 10 min, while continuing stirring. During this time, the agar will solidify and beads entrapping *A. fumigatus* conidia are formed. After 10 minutes, place the flask on ice and continue work inside laminar flow hood for the remaining steps.
    - Add 5 ml of sterile PBS into 6 sterile 50 ml Oak Ridge high-speed centrifuge tubes, and transfer 25 ml of bead/oil suspension into each tube using a 25 ml pipette. Centrifuge at 9,000 xg for 20 min at 4˚C.
    - For each tube, remove the oily supernatant with 25 ml pipette, and gently resuspend the beads with 5 ml of sterile PBS using a glass Pasteur pipette. From this point, all pipetting should be done exclusively with glass Pasteur pipettes. Vortex the bead suspension gently, and transfer to 15 ml Polystyrene conical tubes. Add 10 ml of sterile PBS into each tube, and resuspend the beads by inverting the tubes twice. Centrifuge at 400 x g for 10 min at room temperature.
    - Remove the supernatant and resuspend the beads in 5 ml of sterile PBS. Transfer bead suspension into new 15 ml Polystyrene tubes. Add 10 ml of sterile PBS, and invert tubes. Centrifuge at 400 xg for 10 min at room temperature.
    - Repeat previous step two more times (change tubes every time), but before the final centrifugation, pool all the beads into one 50 ml conical tube. After the final centrifugation, resuspend beads in 10 ml of sterile PBS.
    - Filter the bead suspension through a sterile metal mesh (pore size 280 μm) into a new 50 ml conical tube to ensure that beads used for the experiment are no bigger than 250 μm in diameter. Inspect the beads under a microscope; they should be round and contain conidia inside them. Store beads at 4˚C for up to 48 hours before use.

- Determine the concentration of *A. fumigatus* conidia within the beads by diluting 0.1 ml of beads (use a 1 ml disposable pipette) in 3.9 ml PBS-T. Disrupt the beads by homogenizing for 30 sec. Make serial dilutions (1:10) of the homogenates in PBS. Spread 0.1 ml of each dilution onto YPD agar plates in triplicate, and incubate the plates at 37˚C overnight. Count the colony-forming units (CFUs) the following morning.
- Adjust the final *A. fumigatus* concentration within agar beads stock to 5x10^7 conidia/ml by diluting the bead suspension with sterile PBS.
- Prepare the control agar beads (without *A. fumigatus* conidia) by using the same methods as described above but omitting the *A. fumigatus* conidia (sham beads).

- Mice
  - Use 18-20 g female C57BL/6 mice (or acceptable equivalent). Groups of 8 mice should be used for each experimental condition and compared with a group infected with beads containing a total of 8 mice per infected group plus an additional group of 8 that should be infected with sham beads.
  - All mice should be sacrificed no later than 28 days post-infection as time points longer than this have not been studied.
- Infection of mice
  - Before endotracheal infection, mice should be anesthesized by inhalation of 4% isoflurane.
  - Upon successful anesthesia (no pedal or ocular reflex), install mouse under surgical microscope in the vertical position. Hold the mouse on a restraining board by holding the mouse by its upper incisor teeth with paper clip.
  - Pull the tongue gently to the side of the mouth to reveal the vocal cords. Insert a curved 26-gauge needle into the mouth and guide the needle through the pharynx to gently touch the vocal cords to see the lumen of the trachea. Inject 50 µl of the agarose bead suspension (containing 2.5x10^6 *A. fumigatus* conidia) into trachea to reach the lung. Sterilize the needle with 70% ethanol between each fungal strain.
  - Place back the mice in their cage and they should wake up within 1 minute
- Monitoring mice
  - Monitor mice twice daily for signs of distress, such as:
    - Rapid breathing
    - Breathing very slow, shallow and labored (preceded by rapid breathing)
    - Rapid weight loss due to dehydration
    - Ruffled fur
    - Hunched posture
    - Body temperature less than 28°C.
    - Impaired ambulation (unable to reach food or water easily)
    - Evidence of muscle atrophy or other signs of emaciation (body weight is not always appropriate).
    - Any obvious illness such as signs of lethargy (drowsiness, aversion to activity, physical or mental alertness, anorexia (loss of appetite, especially when prolonged), bleeding, difficulty breathing, CNS disturbance and chronic diarrhea
  - Mice that are moribund should be euthanized humanely using CO_2 asphyxiation or other acceptable means.
  - The experiment can be continued until 28 days after infection

- Mortality should not exceed 10% for the results of the experiment to be interpretable

7. Outcome measures

Histopathology: Beads containing hyphae should be visible from 24hours after inoculation up to 28 days after infection. Hyphae are generally not seen invading the tissues and are restricted to the agar beads.

Fungal burden: Fungal burden can be estimated by determining the galactomannan (GM) content of pulmonary homogenates using the commercial BioRadGM assay. Fungal burden can be measured from lungs in two ways. For fungal burden alone, lungs should be completely homogenized in 5mL of PBS, and then diluted 1:60 for testing in the GM assay kit. Alternately, if pulmonary leukocyte populations are to be examined by FACS or other techniques, lungs can be digested in 10mL RPMI containing collagenase as per standard cell isolation protocols, and an aliquot of the fluid diluted 1:30 in water for GM analysis. The manufaturer's protocol is then followed using these diluted samples.

Temperature: Normal mouse peritoneal surface temperature is around 30ºC (+/- 1ºC). Mice that develop a a body temperature <28ºC for more than 12 hours should be humanely sacrificed.

Weight loss: Usually mice will lose ~10% bodyweight during the course of the experiment with 20% body weight loss being a clinical endpoint.

8. References:

Urb M, Snarr BD, Wojewodka G, Lehoux M, Lee MJ, Ralph B, Divangahi M, King IL, McGovern TK, Martin JG, Fraser R, Radzioch D, Sheppard DC. Evolution of the Immune Response to Chronic Airway Colonization with Aspergillus fumigatus Hyphae. Infect Immun. 2015 Sep;83(9):3590-600. doi: 10.1128/IAI.00359-15.