Marzluff Lab PET/CT Imaging Procedure

Acknowledgements:

This procedure was originally developed by John Marzluff, Donna Cross, Robert Miyaoka, and Satoshi Minoshima. It has been further refined by Ila Palmquist, Toru Shimizu, Barbara Lewellen, Greg Garwin, Adrienne Lehnert, and Loma Pendergraft.

This document was written by Loma Pendergraft, with input from Barbara Lewellen, Adrienne Lehnert, and John Marzluff

Personnel

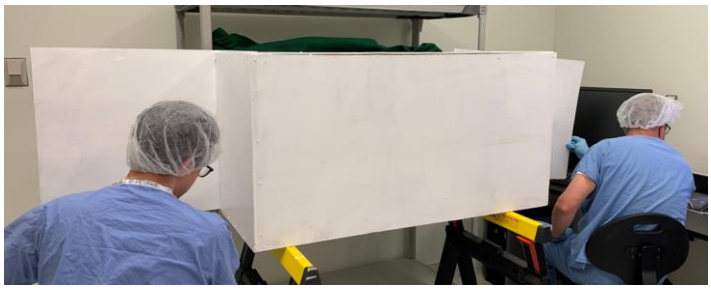
- We always perform the following procedure with three personnel; while we believe it's possible to do with only two people, we've never attempted it. Two experimenters (hereafter referred to as handlers) are responsible for handling the crow- they inject it with FDG-18, expose it to the stimulus, anesthetize it, strap it to bed, and monitor its health during acquisition. The third experimenter is the radiologist- they are responsible for calculating the FDG-18 dose and operating the PET (and assorted) equipment.

Before scan day

- Notes:
    a. Preparing crows for scan
        i. We keep our crows in a protected outdoor aviary during their time in captivity.
        ii. To better acclimate the crows to the scan, we simulate the scan conditions for multiple birds per day (each crow goes through this acclimation every 2-3 days). First, we simulate the injection (capture bird, cover eyes with a cloth, lay on its back, spray belly with water, and lightly pinch the belly at the same location) then place the bird in smaller cage (same dimensions as the holding cages in the scan room) with a "stage" (see equipment below) for approximately one hour before returning them to their regular cage.

    b. Preparing equipment
        i. Because all aspects of the scanning room (such as lights, sounds, personnel present, etc) could potentially affect the bird's attention during the uptake

phase, we constructed a wooden "stage" which, when positioned in front of the holding cage, blocks all view of the room, ensuring that the crow is only being exposed to the target stimulus. The stage is painted with waterproof white paint to meet the facility's decontamination standards and contains an LED light and sliding panels which allow us to alternatively show and hide the stimulus. The paint color, light intensity, and light angle within the stage are kept consistent between trials. We use plastic sawhorses to elevate the stage in front of the stimulus cage.



c. Planning stimulus exposure
    i. Crows are intelligent enough to remember the previous stimulus they were given if they are scanned more than once. If any of the planned stimuli could be considered frightening to the crow (predators, dead conspecifics, alarm calls, etc), the crow will subsequently associate the scanning room/process with the fearful stimulus, which will confound the results if subsequent scans expose the crow to benign stimuli. For this reason, it is important that scary stimuli are presented to the crow last.

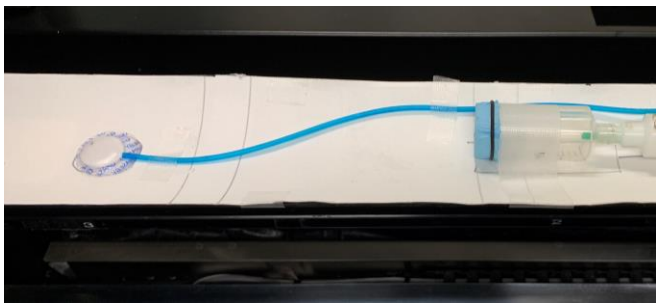
- <u>Handlers</u>
    a. Transporting and housing crows
        i. The evening before the procedure (typically around 1600-1700), we weigh and remove the 1-3 birds that are scheduled to be scanned from their flight cage and use small animal carriers to transport them from the aviary to the facility with the PET scanner.
        ii. The crows are placed individually inside holding cages (0.5 x 0.5 x 1.0 m) and kept overnight in a holding room adjacent to the scanning room. Cages have a perch and access to water, but not food. This ensures that the birds have not eaten anything for at least 14 hours, which is necessary for the body to rapidly absorb FDG. Having an empty stomach also reduces the likelihood of them vomiting and asphyxiating or inhaling food items while under anesthesia.
        iii. Keeping the crows in the facility overnight allows them to acclimate to both the small cages and the sight/sounds of the facility, which should reduce those potential confounding factors from the scan results.
        iv. We always keep the birds in the holding room away from the scanning room until it's their turn to be scanned. This ensures that the crows are not stressed by our activity as we prep the scanning room or progress through a scan with another bird. Additionally, sometimes crows give distress calls when being

handled, so we further reduce the waiting birds' stress levels if they can't hear them.

- Radiologist
    a. Ordering dose
        i. With our supplier, we order enough dose to do 3 birds in one day. This done by calculating 1 mCi per bird at the times we expect to inject each one. The doses are then drawn up from the delivered syringe. The volume we receive is less than .25 ml. From this the first dose is drawn. Saline is added to bring the volume to .050 ml. Then all other doses are drawn just before injection time and QS up to the correct volume.

Before procedure

- Radiologist
    a. The scanner is set up the day of the scan
        i. All QC (calibration) protocols for the scanner are done first
        ii. Scan folder is created. Inside of it is a Data folder, Acquisitions and reconstruction folders
        iii. The extended bed with extra length is put on the moveable platform.
        iv. On the scan bed an assonant, non-dust paper is fitted to the bed. The paper has marking for placement of the nose cone, scan area and respirator pad for the bird. These items are then put on the table.



        v. Isoflurane levels on the vaporizer for both the scan table and the induction box are checked and topped off as is needed
        vi. O-2 supply is checked

- Handlers
    a. The handlers bring all the items which are not kept at the facility (batteries, tablet and camera, food for the crows after the procedure is complete, etc).
    b. Approximately 20-30 minutes before beginning the scan, we cover a crow's cage with a cloth and transport it to the scan room. This allows the bird time time to acclimate to the sounds of the scanner and experimenters.
    c. All personnel double-check to ensure that all required tools and equipment are present and in their correct position.

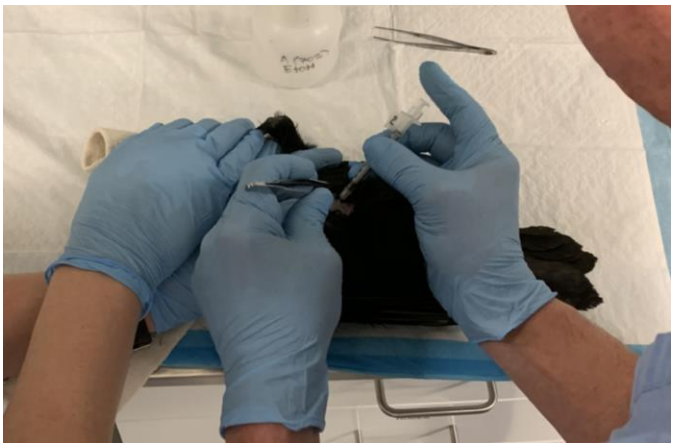
FDG-18 Injection

- Notes:
    a. We use [F-18] fluorodeoxyglucose (FDG-18) for our radiotracer,

b. We administer the radiotracer via intraperitoneal injection. The rate of uptake (and subsequent timing of various tasks) are dependent on this method of injection- FDG is absorbed at a different rate when injected intravenously. See figure S1 in Marzluff et al. 10.1073/pnas.1206109109 for FDG-18 activity within a single crow after intraperitoneal injection.
c. With PET imaging, we must find a balance between maximize the crow's exposure to the stimulus during uptake (more FDG-18 can concentrate in the brain, giving more contrast between the high/low activity regions) without compromising our ability to detect/record FDG-18 within the brain (which peaks around 23 minutes after injection), all while budgeting the proper amount of time for prepping the crow for the scan (too much and you sacrifice stimulus time, too little and you don't have a buffer zone for small delays). For our more recent experiments, we have found the following timeline offers a good compromise:
    i. 0 min: inject crow with FDG-18 (start timer)
    ii. 3 min: begin exposing crow to stimulus
    iii. 13 min: stop the stimulus and begin prepping bird for scan (if nothing goes wrong, we are usually finished by the 23 minute mark- we have 3 minutes of buffer time should we run into any problems)
    iv. 26 min: begin PET acquisition

- Radiologist
    a. After we are satisfied that everything is ready, the radiologist draws a dose of 1 mCi FDG-18 with a volume of .050 ml. The time and activity is recorded in the log book
    b. The dose is consistent for all birds in the study. The bird's weight is recorded and adjustments for the images to be able to compare bird to bird is made after the study.
- Handlers
    a. After the radiologist returns with the dose of FDG-18, the handlers capture the crow from the holding cage. We cover the bird's eyes with a cloth as soon as possible to limit its view of the room and experimenters.
    b. One handler places the bird on its back and holds it in place. The bird's feet and wings are held out of the way such that the second handler has full access to the crow's belly.
    c. The second handler parts the feathers around the belly and sprays the exposed skin

with a disinfectant (70% alcohol). This handler then uses forceps to grasp and pull a small fold of skin outwards and injects the FDG into the bird's peritoneum. By lifting the skin around the injection site and inserting the needle slowly, we minimize the risk that the needle accidentally punctures the intestines.



d. At the moment of FDG-18 injection, the handlers inform the radiologist to start the timer (see Notes above for time markers). The start time is recorded in the logbook.
e. After injection, one handler returns the bird to the holding cage and re-covers the front with a cloth. The other handler notes the time of injection on the datasheet and turns on the video camera for remote viewing of the crow's activity within its cage during the stimulus exposure.

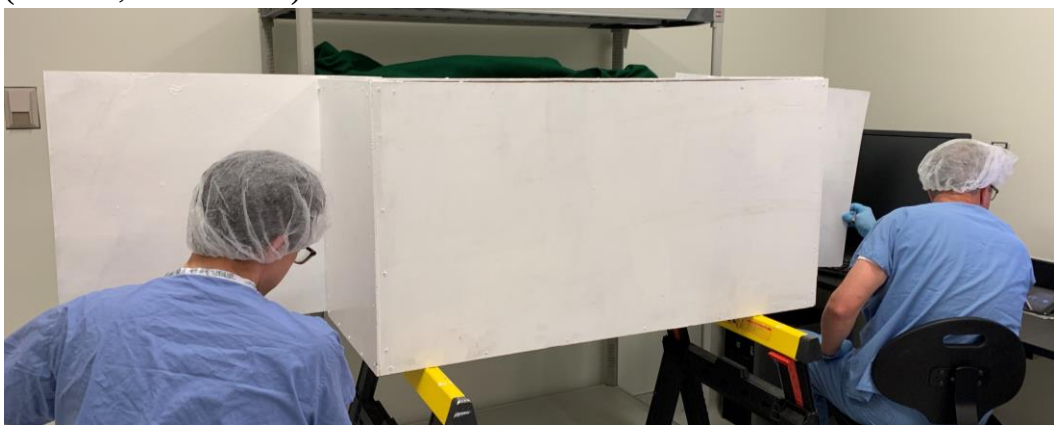
Stimulus

- Notes:
    a. We use a wireless camera (GoPro) and a synchronized tablet to simultaneously record and monitor the bird's activity during the stimulus phase.
    b. It is important to re-stimulate the birds repeatedly during the stimulus phase (rather than leaving the stimulus in view the entire time) so that the bird does not get unfocused and start paying attention to other aspects of the stage/room. We accomplish this by using sliding panels to alternatively hide/reveal the stimulus within the stage. Because the crow's cage is still mostly covered with a cloth, the bird is plunged into darkness whenever the stage panels are closed. The sudden contrast between the dark cage and the well-lit stimulus ensures the crow is extra attentive whenever the panels are opened.
    c. The duration of the stimulus shown/hidden time blocks during the 10 min stimulus phase can be flexible but should be consistent for all birds/trials within an experiment. We use 60s/30s time blocks (see below)
- Radiologist
    a. As the crow is being returned to its cage, the radiologist collects the empty FDG-18 syringe and returns it to the hot lab
    b. The syringe is measured in the dose calibrator and the resulting residual is measured with the activity and time recorded in the logbook. This is how we know the exact amount of FDG-18 that was injected.

c. The radiologist quietly re-enters the scan room (if the crow is currently being exposed to the stimulus, the radiologist waits until the panels are shut before entering).

- Handlers
  a. After the crow is returned to its cage, the handlers move the stage in front of the cage. After verifying that the stage panels are shut (and the bird cannot see the stimulus inside), the handlers lift the cloth from the front of the cage, so that the crow can see the exterior panels of the stage.

  b. At 3 minutes post-injection, both handlers use the panels to alternatively show (open panels for 60s) then hide (close panels for 30s) the stimulus for 10 minutes (7 shows, 6 hides total).

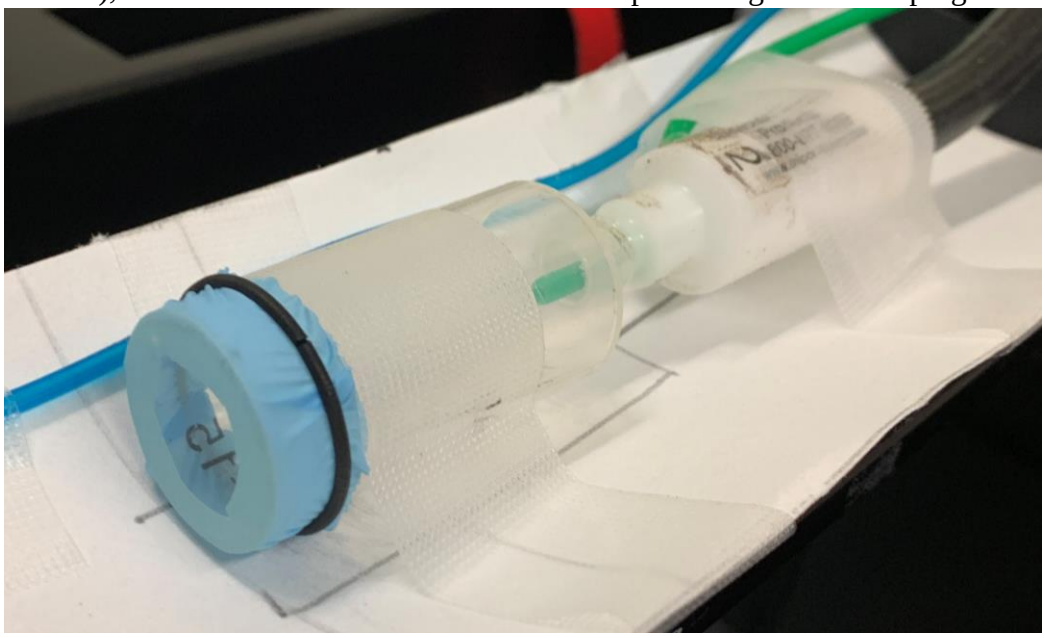


  c. One handler uses the tablet (synchronized to a wireless video camera) to observe the crow's behavior within the cage during the stimulus phase. This handler

records any unusual or interesting behaviors (panting, pacing, craning neck to see stimulus, etc) on a data sheet- it is especially important to note if the crow is watching the stimulus through one or both eyes (and which eye if one), as that affects which of the two brain hemispheres is most active.

Anesthetizing the bird

- Notes
    a. We anesthetize crows using vaporized isoflurane in oxygen with a flow rate of 300-800 mL/min.
    b. Rather than intubating the birds (which is difficult to do correctly and can lead to injuries or complications), we created a crow-sized anesthetic nose cone using a 50-mL syringe tube and latex film (obtained by cutting a section from a disposable latex glove). We cut the barrel of the syringe in half along its axial axis, then stretched the latex over the cut opening. We cut a smaller hole into the latex film and attached the anesthesia lines to the syringe's adaptor. The crow's beak can be inserted into the syringe through the hole in the latex film (past the nostrils), which forms a seal around its beak and prevents gas from seeping out.



    c. We initially induce crows at 5% isoflurane (knocks them out rapidly), but then reduce isoflurane concentration to 2.5-3% to keep them unconscious during the scan.
- Radiologist
    a. During the last stimulus exposure (12 minutes post-injection), the radiologist turns on the anesthesia at the inducing station.
- Handlers
    a. After the final stimulus exposure (13 minutes post-injection), the handlers close the stage panels, re-cover the front of the cage with the cloth, then move the stage out of the way.

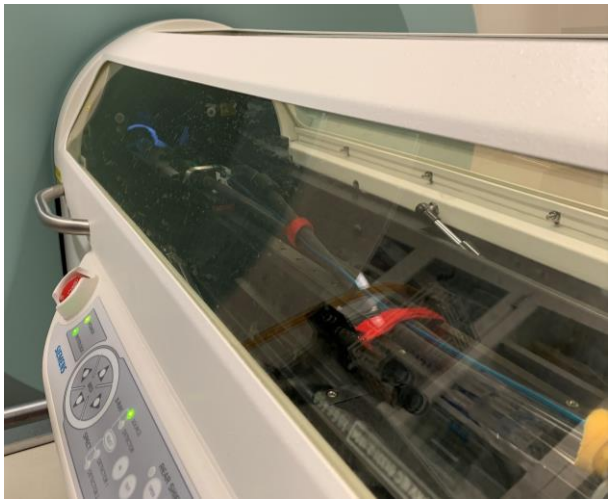
b. One handler captures and extracts the crow from the cage, while the second handler covers the bird's eyes with a cloth and places/holds the bird's bill into the anesthetic mask.
c. As the crow is anesthetized (and becoming more relaxed), the first handler moves the crow's legs behind it, as though it was lying flat on its belly (this makes it easier to place the crow on the bed when we move it). The second handler periodically (every 10 s) checks the crow's eyes- once both eyes are closed and the crow is completely relaxed, it is fully unconscious. However, it is not uncommon for birds to convulse briefly (flapping wings and moving legs) as they enter sleep.

Strapping the unconscious crow to the bed

- Notes:
    a. We use Velcro straps and taped gauze to secure the crow's head, torso, and wing tips/feet to the bed. Velcro is easier to apply and does not restrict the bird's breathing as much as a sock or jacket.
- Radiologist
    a. As the crow is anesthetized at the inducing station, the radiologist turns on the anesthesia at the multimodality bed.
    b. After the crow is moved to the bed, the radiologist activates the breathing sensor and informs the handlers of the signal quality. Afterwards, the radiologist assists the handlers.
- Handlers
    a. Once the crow is fully unconscious, the first handler quickly moves the crow to the multimodality bed and inserts the crow's bill into the bed's anesthetic cone. The second handler removes the cloth from the crow's head, turns off the isoflurane inducer, then assists the first handler in securing the crow to the bed. They align the crow and secure the bird with 2 Velcro straps (one at terminal end

of wings, one around breast) and a cotton gauze taped across the neck to stabilize the head.

b. The second handler and/or the radiologist examine the alignment of the crow's head from the front of the bed and inform the first handler how to adjust the head position so that it's straight and level.



c. After everyone is satisfied with the crow's position, the handlers move the bed into the proper position within the machine, use additional Velcro to strap the lose cables (anesthesia and breathing sensor) to the bed, and close the seal.

Starting PET/CT acquisition

- Notes:
    a. We monitor the bird's condition using a breathing sensor. The respiration rate of anesthetized crows can vary widely (typically ranging between 27 and 45 breaths/min) depending on the individual but should remain stable throughout the procedure. Sometimes, crows take an extra deep breath, or lie unevenly on the breathing pad, both of which can disrupt the signal but are not causes for alarm. The major thing to pay attention to is a *change* to the respiration rate: if the bird's respiration rate suddenly starts increasing within 5 minutes of being induced, that indicates that it's starting to wake up and we need to increase the isoflurane concentration slightly (we return the isoflurane to 3% after the respiration rate

slows down again). However, if the crow has been unconscious for longer than 5 minutes and its respiration rate suddenly increases, that indicates that the bird is in danger and we need to immediately *decrease* the isoflurane concentration. If the bird stops breathing, *immediately* abort the scan, turn off the isoflurane (but keep the pure oxygen flowing), and begin doing chest compressions on the bird (keep their beak in the oxygen mask).

b. We acquired high-resolution images using a Siemens Inveon PET/CT system, which consists of co-registered PET and CT scanners. The scanners share a bed, and have a bore diameter of ~12 cm. One PET field of view is approximately 8 x 13 cm^2

c. The scanner we use was designed for rats, so it is a tight fit for the larger crows- sometimes a strap, shoulder, or leg catches on something as the bed moves the unconscious bird into the acquisition field. To avoid this, we use a flashlight to monitor the bird as it moves through the machine and must sometimes reach in with a tool (a ruler works great for this purpose- it's long, thin, and semi-rigid) and prod/adjust part of the body before it snags on something.

- <u>Handlers</u>
    a. The handlers are responsible for monitoring the crow's breathing.
    b. Whenever the bed moves through the machine, the handlers monitor the bird's progress and stand ready to intervene if it looks like part of the crow is going to snag on something inside the machine.
- <u>Radiologist</u>
    a. After the bird is secured to the multi-modality bed, the CT shield door is lowered, and the radiologist starts the image acquisition workflow.

b. A CT scout image is collected to verify the positioning of the crows' brain the center of the field of view.
c. A 15 min PET acquisition is started 24 min post-injection. The list-mode data will later be edited to keep only 10 min of data starting at 26 min post-injection.
d. After the PET is completed, a 2-bed position CT image is collected for attenuation and scatter correction as well as for anatomical reference in the PET image.
e. The CT image is reconstructed with the following settings: FBP algorithm, no downsampling, slight noise reduction, Shepp-Logan filter, a "Rat" beam hardening correction and correctly calibrated HU scaling factor.
f. The PET data is histogramed dynamically and the correct time segment (26-36 min post-injection) is isolated
The PET image is reconstructed using the following settings: 3D OSEM with 2 iterations and 18 subsets, scatter and attenuation corrections, in a 128x128 matrix

Post-procedure recovery

- Notes:
  a. After all crows have been scanned, they are given food.
  b. The crows must remain in the facility overnight (so that they are no longer radioactive) after the procedure before they are returned to their regular cages in the aviary.
- Handlers
  a. Approximately 20 minutes prior to scan completion, the handlers move the current bird's cage back to the holding room and, if applicable, move the next crow's covered cage into the scanning room (see Before Procedure above).
  b. Approximately 2 minutes from scan completion, we lower the isoflurane concentration to 2%. 1 minute from scan completion, we lower it further to 1%. This causes the bird to regain consciousness more rapidly after the scan is finished.
  c. When the scan is completed, one handler turns off the isoflurane but allows the bird to continue breathing oxygen (decreases the crow's wake up time). Once its eyes open, the first handler carries the crow to the second handler, who holds the crow over a disposable medical pad (chux) and supports its head as it wakes up. Rarely, the crow might vomit or produce feces as it regains

consciousness- the chux protects the handler from additional radiation exposure.

d. Once the crow has regained the ability to grip with both legs (signifying that it can perch with minimal risk of falling and injuring itself), it is returned to its cage in the holding room.

- Radiologist
    a. After the scan is finished and the crows are in recovery, the rooms are cleaned and surveyed for radiation spills. All log books are updated
    b. PET and CT images are reconstructed and exported to DICOM format for analysis

We will cover the details of preparing/analyzing the PET images in a separate document (coming soon)