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Continuous Blood Sampling in Small Animal Positron Emission Tomography/Computed Tomography Enables the Measurement of the Arterial Input Function

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TITLE:

Continuous Blood Sampling in Small Animal Positron Emission Tomography/Computed Tomography Enables the Measurement of the Arterial Input Function

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KEYWORDS:

PET imaging, kinetic modeling, quantification, radiotracer uptake, continuous blood sampling, arterial cannulation, arteriovenous shunt, arterial input function, small animal, rodent, rat

SUMMARY:

Here a protocol for continuous blood sampling during PET/CT imaging of rats to measure the arterial input function (AIF) is described. The catheterization, the calibration and setup of the system and the data analysis of the blood radioactivity are demonstrated. The generated data provide input parameters for subsequent bio-kinetic modeling.

ABSTRACT:

For quantitative analysis and bio-kinetic modeling of positron emission tomography/computed tomography (PET/CT) data, the determination of the temporal blood time-activity concentration also known as arterial input function (AIF) is a key point, especially for the

characterization of animal disease models and the introduction of newly developed radiotracers. The knowledge of radiotracer availability in the blood helps to interpret PET/CT-derived data of tissue activity. For this purpose, online blood sampling during the PET/CT imaging is advisable to measure the AIF. In contrast to manual blood sampling and image-derived approaches, continuous online blood sampling has several advantages. Besides the minimized blood loss, there is an improved resolution and a superior accuracy for the blood activity measurement. However, the major drawback of online blood sampling is the costly and time-consuming preparation to catheterize the femoral vessels of the animal. Here, we describe an easy and complete workflow for catheterization and continuous blood sampling during small animal PET/CT imaging and compared it to manual blood sampling and an image-derived approach. Using this highly-standardized workflow, the determination of the fluorodeoxyglucose ([^{18}F]FDG) AIF is demonstrated. Further, this procedure can be applied to any radiotracer in combination with different animal models to create fundamental knowledge of tracer kinetic and model characteristics. This allows a more precise evaluation of the behavior of pharmaceuticals, both for diagnostic and therapeutic approaches in the preclinical research of oncological, neurodegenerative and myocardial diseases.

INTRODUCTION:

Positron emission tomography/computed tomography (PET/CT) is a nuclear imaging technology that enables visualization of metabolic processes in the body following injection of a radioactively labeled ligand, also called tracer. Whereas the ligand is a molecule that is involved in a metabolic pathway or targets cell surface proteins, the radioactive label is a positron-emitting radionuclide. Gamma rays are indirectly emitted by the positron decay and allow the detection of its distribution in the organism with extracorporeal PET detectors. In this way, different cellular molecules can be targeted: neurotransmitter receptors and transporters, metabolic processes like glycolysis or mitochondrial proteins like the translocator protein 18 kDa (TSPO) to detect activated glia cells.

In preclinical research, PET/CT is an attractive method to study biochemical processes in a non-invasive manner in vivo, thus allowing longitudinal studies. PET/CT data support the analyses of disease mechanisms, the assessment of the characteristics and pharmacokinetics of new drugs and the validation of both, current and novel radiotracers for translational research.

During PET/CT analyses three tracer states can be defined (example of the 2-tissue compartment model): First, the tracer flows within the blood after its application (state 1; $conc._{[blood]}$). Second, it enters the tissue via the capillary bed and can there either freely move within the extracellular space or is unspecifically bound to diverse cellular or extracellular structures (state 2; $conc._{[unspec]}$). Third, the tracer can be specifically bound (with or without metabolic trapping) to its target molecule (state 3, $conc._{[spec]}$). All these dynamic processes between the compartments are to some extent bidirectional and the diffusion processes are described by rate constants (K1, k2, k3, and k4). While the concentration of the tracer in the blood (i.e., state 1) is called “Input”, the concentration of unspecifically and specifically bound tracer (i.e., state 2 and state 3) is called “Output” and can be directly derived from the PET Image. This physiological relation can be displayed in the 2-tissue compartment model (**Figure**

1).

[Place Figure 1 here]

In the ideal case, conc.$_{[spec]}$ is proportional to the concentration of its target molecule. However, the output of the PET/CT measurement is the sum of conc.$_{[spec]}$ and conc.$_{[unspec]}$. To determine conc.$_{[spec]}$ in the region of interest, in parallel the conc.$_{[unspec]}$ of a reference region devoid of the target protein/pathway is determined. By using appropriate mathematical equations one can now calculate conc.$_{[spec]}$, most commonly using the compartment model (a bio-kinetic modeling approach). However, in many cases, such a reference region devoid of the target protein is not available[1,2]. In these cases, the conc.$_{[blood]}$ can be used to determine conc.$_{[spec]}$. Since the conc.$_{[blood]}$ is varying due to different liver and kidney clearance, excretion, blood flow, different brain-blood barrier penetration and disease-related factors[3], the current gold standard is to measure the conc.$_{[blood]}$ in parallel to the PET/CT scan by continuous blood sampling. This gives the arterial input function (AIF), which is defined as conc.$_{[blood]}$ over time[4]. Of note, performing continuous blood sampling is considered technically highly challenging, especially in small animals such as rats or mice[5].

Here, we provide an easy and practical protocol to continuously sample blood from rats via an arteriovenous (a-v) shunt between the femoral vein and artery. Coupled to a commercially available detector-pump system, we are able to generate a real-time, continuous AIF during dynamic [^{18}F]fluorodeoxyglucose ([^{18}F]FDG)-PET/CT scans in rats and compared it to alternative approaches. PET/CT imaging was performed in male sprague dawley rats at an age of 4 months with an average weight of 462 g ± 33 g (mean ± standard deviation) using a multimodality PET/CT scanner.

Since a wide variety of devices is used during the series of measurements (dose calibrator, online blood sampler, PET/CT, and well counter), a quality control procedure referred as cross calibration is needed to check the quantitative accuracy of all systems and to compensate for differences. Cross calibration in the context of online blood sampling means that the count rate for a given activity concentration measured in corrected PET images can be converted into the concentration measured with the twilite system for the same concentration. Therefore, a cross calibration procedure between PET/CT, blood sampling system, and well counter has been established.

This highly standardized methodology provides a powerful approach to quantify metabolic and cellular processes in preclinical small animal research and is an elegant way to improve the reliability and reproducibility of the AIF. The AIF can then be used to quantify the specifically bound tracer in tissue in preclinical PET/CT data using bio-kinetic modeling.

PROTOCOL:

All animal handling and experiments were approved by the state Animal Research Committee of Mecklenburg–Western Pomerania (LALLF M-V/7221.3-1.1-004/18, approval: 03.04.2018). The experiments were performed in compliance with the ARRIVE Guidelines.

NOTE: Animals were kept under standard conditions (22 ± 2 °C, 12 h day-and-night cycle) with water and food ad libitum. All needed equipment for the preparation of the shunt system, the operation procedure and the actual measurements are listed in the **Table of Materials**.

1. Preparation and surgical procedure for catheterization of the animal

1.1. Fast the animal for at least 12 h with free access to water. For anesthesia, place the rat in an induction chamber and fill it continuously with oxygen/isoflurane mix. For initiation use 2.5−3.5% isoflurane and for maintenance 1.5−3.0% (flow rate of 1.2−1.5 L/min).

NOTE: Fasting is necessary for studies using the tracer [^{18}F]FDG but not for other tracers. Measuring glucose blood levels using manual blood draws described in section 4 is recommended to ensure stable values or to correct for in kinetic modeling.

1.2. Place the anesthetized rat in dorsal position on a heating mat, under the surgical microscope and add vet ointment on its eyes. Monitor and maintain the body temperature of the rat continuously during the experiment (37 ± 0.5 °C) with a rectal probe.

1.3. Tape the legs of the rat to the work surface to hold the legs in position. Disinfect the operating site with a mucosal disinfectant and shave the leg and crotch (operation side) of the rat. Finish with a final cleansing with the disinfectant.

1.4. Make an incision of about 20 mm using surgical forceps and scissors at the groin of the rat. Dissect the fine skin layers and expose the femoral vein, artery and nerve with the micro forceps. Place two fine filaments under each femoral vein and artery.

1.5. Seal vein and artery with each distal filament and hold under tension with a bulldog clamp. Use the proximal suture filaments to tension the vessel using the bulldog clamps (without a knot).

1.6. Block the vein with an aneurysm clamp proximal, but 2−3 mm distal from the suture with the bulldog clamp. Use corneal scissors to make a small incision into the vein (1/3 of the diameter) and remove leaking blood with a sterile cotton swap. Dilate the vein with a dull forceps and hold it open. Insert the sharpened catheter (inner diameter [ID]: 0.58 mm, outer diameter [OD]: 0.96 mm) into the vein and push it in proximal direction, up to the aneurysm clip.

1.7. Open the aneurysm clip and push the catheter further in proximal direction (approximately 2−3 cm), if the catheter is placed right, blood will flow into the catheter. Secure the catheter with the proximal suture by making two knots; if necessary, place an additional suture around the vein and the catheter. Check the functionality of the catheter by flushing and aspirating with an insulin syringe (30 G needle) filled with 100 µL of heparinized saline solution (50 units/mL).

1.8. Place the catheter in the artery by repeating steps 1.6 and 1.7.

1.9. When both catheters are correctly placed, close the leg with sutures and carry the animal to the PET/CT.

NOTE: Be as careful as possible with the catheters during transport of the animal, otherwise shifting of the catheter might occur.

2. Setup of the shunt system

[Place Figure 2 here]

2.1. Cut off 6 parts of the fine bore polythene tubing (FBPT) (ID: 0.58 mm, OD: 0.96 mm) with a length of **c** = 735 mm; **e** = 100 mm; **f** = 171 mm, **g** = 875 mm; **h** = 90 mm and **i** = 75 mm (**Figure 2**). Cut off 8 parts of the silicone pump tubes (black/black/black, ID: 0.76 mm, OD: 2.48 mm) with a length of approximately 20 mm.

2.2. Place reduction connectors (from ID 2.5 mm to ID 1.5 mm) on both ends of the silicone pump tube **d** (yellow/blue/yellow, ID: 1.52 mm, OD: 3.20 mm). Put a prepared 20 mm part of the silicone tubes (black/black/black) on the other end of the used reduction connectors (see **connector blue** in **Figure 2**).

2.3. Place the prepared part **c** of the FBPT in the assembled **connector blue** on the one end of the silicone pump tube **d** (yellow/blue/yellow) and the prepared part **e** of the FBPT in the assembled **connector blue** on the other end. Put a prepared 20 mm part of the silicone tubes (black/black/black) on the ends of two T-pieces **5 and 6** (tube T-connector ID: 1.5 mm; see **connector green** in **Figure 2**).

2.4. Connect the free end of part **e** of the FBPT to the left side of the assembled **connector green (5)** and place the prepared part **f** of the FBPT at the opposite side of **connector green (5)**. Place the free end of part **f** of the FBPT in left side of assembled **connector green (6)** and place the prepared part **g** of the FBPT at the opposite side of **connector green (6)**. Add the prepared part **h** of the FBPT to the free end of the assembled **connector green (5)** and the prepared part **i** of the FBPT to the free end of the assembled **connector green (6).**

2.5. Connect a combi-stopper to a hypodermic needle (G 23 x 1 1/4''/ø 0.60 mm x 30 mm) and add it to a three-way valve. Place the prepared three-way valve with the needle in the free end of the part **h** of the FBPT. Connect a combi-stopper to a hypodermic needle and place the needle in the free end of the part **i** of the FBPT.

NOTE: Before starting the online blood sampling, see section 5.

2.6. Put the free ends of part **c** and **g** of the FBPT into a 100 mL beaker filled with 20 mL of

heparinized saline solution (50 units/mL). Start the peristaltic pump with a flow rate of 1.52 mL/min so that the shunt system is completely filled with the physiological saline solution. Afterwards set three scissor clamps at the ends of part **c** and **g** and in the middle of part **i** of the FBPT.

2.7. Release the scissor clamps from part **c** and **g** of the FBPT. Connect the arterial catheter **a** to the free end of part **c** of the FBPT and connect the venous catheter **b** to the free end of part **g** of the FBPT (see **connector orange** in **Figure 2**).

3. Image acquisition and reconstruction

3.1. Place the animal in head-prone position on the shuttle bed pallet (70 mm). Control respiration of the rat and keep body temperature at 37 ± 0.5 °C using a heating pad and a rectal probe throughout image acquisition. Move the shuttle bed to the extended bed position for injection (pre-acquisition) and connect the inserted catheters to the shunt system.

3.2. Keep the animal under anesthesia with isoflurane (2.5% isoflurane in oxygen, flow rate 1.2−1.5 L/min) via a nose cone.

3.3. Start the peristaltic pump with a flow rate of 1.52 mL/min to fill the shunt system with the blood of the animal. Move the shuttle bed to the center of the field of view of the PET detection ring and start the online blood sampling system (see section 5).

3.4. Start the PET/CT workflow using parameters described in section 3.5 after 60 s and subsequently inject a dose of approximately 22 MBq [^{18}F]FDG in a volume of about 0.5 ± 0.1 mL intravenously via the T-piece. Flush the T-piece with about 150 µL of heparinized saline solution afterwards.

3.5. Acquire a dynamic PET over 60 min and a CT scan at the end of the PET imaging.

3.5.1. For PET emission acquisition, set 3600 s (60 min) in the **acquire by time** option. Select F-18 as **study isotope** and use 350–650 keV as **energy level** and 3,438 ns as **timing window**.

3.5.2. For CT acquisition, select attenuation scan in the **acquisition** option. In the projection settings field, choose 180 **projection** for a half **total rotation**. For field of view (FOV) and resolution settings, select low as **magnification** and 4 x 4 as **binding** with 275 mm **axial scanning length** and 3328 px as **transaxial CCD size**. In the exposure settings field, set 500 µA for **current**, 80 kV for **voltage** and 180 ms for **exposure time**.

3.5.3. For PET emission histogram, set a series of 20 frames (6 x 10 s, 8 x 30 s, 5 x 300 s and 1 x 1800 s) as **dynamic framing**. Select subtract as **delays**. Choose in the advanced settings field 128 as **sinogram** width, 3 as **span**, 79 as **ring difference and dead time correction**.

3.5.4. For PET reconstruction, use two-dimensional ordered subset expectation maximization

(2D-OSEM) with a generate, apply and save **scatter sinogram**, 4 **iteration** and Fourier for rebinning as **reconstruction algorithm**. Select 128 x 128 as **matrix size** and use 1 as **image zoom**, all as **frames** and all as **segments**.

4. Procedure of manual blood sampling

4.1. Perform manual blood sampling 30 s, 60 s, 90 s, 600 s and 1800 s after starting the imaging acquisition.

NOTE: Increasing the number of manual blood draws especially within the first minute after tracer injection is highly recommended if possible. Therefore the blood sample volume has to be reduced to 20–30 µL per sample[6].

4.1.1. Open the first three-way valve and collect 100 µL of arterial blood into a capillary blood collection EDTA tube 30 s after tracer injection. Repeat for the other time points. Determine the weight of the empty tube and blood filled tube.

4.1.2. Measure the activity (counts/time unit) of the whole blood for 180 s in a well counter, which is later cross calibrated to obtain data in kBq/mL. Record the start time of the well counter measurement. Calculate the activity of whole blood for each time point of the manual blood sampling in kBq/mL, apply decay correction and transfer the data in a time activity curve.

5. Procedure of the online blood sampling

5.1. Place the tube into the detector using the tube guide. Start the blood sampler software (e.g., PSAMPLE) and open the acquisition interface. Ensure that the computer of the online blood sampling setup and of the PET/CT is time synchronized.

5.2. Press the start button exactly 60 s before the tracer is injected to acquire enough data for background correction. Save the raw data via the save button in the PMOD database after the measurement.

5.3. For correction and calibration of the online blood data, switch to the correction interface. Enable the decay correction and select 18 F. Define the start time of image acquisition and enable the average button to perform background correction. Activate the calibration and type in the previously determined calibration factor (see section 7.1).

5.4. Save the corrected and calibrated blood data using the save TAC button and choose the file **blood.crv**. This file can then be loaded as whole blood input curve into the kinetic modeling tool and kinetic modeling can be performed. Decouple the catheters from the extra corporal shunt system.

5.5. Detach the animal from the PET/CT scanner and euthanize with pentobarbital.

NOTE: In this experiment, animals were euthanized after the measurements as brains were used for in vitro analyses in the experimental design. With this setup, repeated measurements in longitudinal studies are also implementable[7]. Use a completely new tube system for the next animal.

6. Image derived Input function

6.1. Open the **Fuse it** tool on PMOD. Load the PET Image as input and the CT as reference. Click already matched.

6.2. Open the voxel of interest (VOI) tool. Place the cursor within the ascending aorta in the CT. Click predefined spherical VOI. Define a radius of exactly 0.7 mm. Extract the time activity information with the VOI statistic button and copy the averaged values to clipboard.

7. Procedure of cross calibration of the twilite system, PET/CT and well counter

7.1. Twilite-PET/CT-calibration

NOTE: The presented workflow for calibration of the twilite is partly based on the procedures described in the reference manual of the PSAMPLE module of PMOD.

7.1.1. Fill a syringe with approximately 100 MBq of [^{18}F]FDG. Measure the exact activity A_F with a calibrated dose calibrator and document it together with date and time of the measurement and the volume of the full syringe. The recorded time is the reference time point for all decay corrections to be performed.

7.1.2. Fill a beaker with 500 mL of tap water. The exact volume is determined by the weighing method. Measure the weight m_e of the empty beaker with an appropriated and calibrated precision scale (at least accuracy class II). Fill the beaker with the tap water and measure the weight m_f of the full beaker.

7.1.3. Calculate the volume V_b of the beaker by using the difference of the mass and the density of tap water (ρ = 0.998 g/mL at 20 °C):

$$V_b = \frac{m_f - m_e}{\rho}$$

7.1.4. Inject the [^{18}F]FDG into the filled beaker and refill the empty syringe to its original volume with inactive tap water and measure the activity A_E of the refilled syringe in the dose calibrator. The activity concentration c_b of the solution in the beaker is given by $c_b = \frac{A_F - A_E}{V_b}\ [kBq/mL]$, which should be approximately 200 kBq/mL.

7.1.5. Fill a 50 mL conical centrifuge tube with the solution from the beaker (avoid large air bubbles) and place it centrally in the field of view of the PET/CT scanner. Fill a catheter identical

to the type used in the PET/CT imaging experiment and place it in the tube guide of the twilite system. Fill the catheter with the tracer solution from the beaker using the peristaltic pump.

7.1.6. Start the measurement of the time activity curve as described in section 5, using the same parameter for integration time, and rebinning as in the experiment, without a catheter guide inside the measurement head. This step ensures the acquisition of enough data for appropriate background correction. After 2 min, without stopping the data acquisition of the twilite system, place the catheter guide with the filled tube into the measurement head, and continue the data acquisition for about 5 min.

7.1.7. Start a 10 min PET acquisition of the 50 mL conical centrifuge tube in parallel followed by a standard CT acquisition for attenuation correction. Reconstruct a static PET image of the 50 mL conical centrifuge tube using the same PET reconstruction algorithm and parameters described in section 3. Use a post processing imaging tool (e.g., PVIEW) and place a cylindrical VOI covering approximately 70% of the volume inside of the reconstructed PET images of the 50 mL conical centrifuge tube. Extract the mean activity concentration c_{PET} in kBq/mL within the VOI.

7.1.8. Go back to the blood sampler software and use the calibration mode to correct the acquired TAC for decay, branching fraction and background. Add all the necessary information for nuclide, activity concentration and the PET acquisition start time. Internally, the software extracts the count rate measured with the twilite system ($CR_{twilite}$) and calculates the cross calibration factor for PET and twilite system ($CF_{PET/twilite}$):

$$CF_{PET/twilite} = \frac{c_{PET}}{CR_{twilite}} \left[\frac{kBq/mL}{cts/s}\right]$$

NOTE: It is important that the same isotope is used for both calibration and PET/CT experiments, as the branching fraction varies between the different isotopes, which is corrected for in the PET reconstruction process. This procedure has to be repeated regularly in terms of quality control, if important components of the system are changed (e.g., tubes, acquisition and reconstruction parameters) and after repair works.

7.2. PET/CT-well counter calibration

7.2.1. To calculate the calibration factor $CF_{well\text{-}counter}$ of the well counter, use the same activity solution that has been produced in the beaker for the calibration of the twilite system. Wait approximately 6 h to allow reduction of specific activity by decay to minimize dead time effects of the scintillation detector of the well counter. Lid the beaker to avoid evaporation.

7.2.2. Calculate the exact time difference to the reference time point and determine the actual activity concentration $c_b(t_+)$ of the solution of the beaker by decay correcting the original activity concentration c_b. Pipette predefined volumes (V_{sample}) that are identical to the volume of the blood samples measured within the experiments (e.g., 200 µL), from the beaker into five

safe-lock tubes. Measure the activity of each of the five tubes with the well counter for 180 s.

NOTE: If the coefficient of variation for a single measurement is greater than 1%, the measurement time should be increased. Record the measured count rate in counts per minute [cpm] for each tube and the measurement start time. Perform a decay correction.

7.2.3. Calculate the calibration factor $CF_{well\text{-}counter}$ for each measurement by dividing the decay corrected count rate $CR_{well\text{-}counter}$ of the well counter by the decay corrected activity concentration of the beaker $c_{beaker(t+)}$:

$$CF_{well-counter} = \frac{CR_{well-counter}}{c_b(t_+) \cdot V_{sample}} \left[\frac{cpm}{Bq}\right]$$

7.2.4. Average the five calibration factors to obtain the mean calibration factor.

REPRESENTATIVE RESULTS:

The setup of the shunt system is displayed in **Figure 2**. Representative results of the continuous blood sampling data compared to manual blood sampling data in three wildtype rats over a time span of 30 min are presented in **Figure 3A,C**. At the beginning of the continuous blood sampling, an initial peak (maximum of radioactivity concentration) can be seen about 30 s after tracer injection. Afterwards, the activity in the blood declines rapidly and reaches a plateau at about 15 min. In the manual blood sampling data the detected Peak is smaller and the plateau is not easily to define (**Figure 3A,C**). The comparison of the continuous blood sampling to the image-derived data is displayed in **Figure 3B,D**. In the image-derived data, the peak and the starting point of the plateau are clearly visible, nevertheless the maximum of the peak is smaller compared to continuous blood sampling data for all animals (**Figure 3B,D**).

A sub-optimal outcome of continuous blood sampling with our setup is shown in **Figure 3E,F**. At the beginning of the continuous blood sampling, no data acquisition within the first 3.5 min was possible due to blood clotting. By disconnecting the tube system at **connector orange** and floating with heparinized saline solution, the flow in the tube system was restarted and the measurement continued. A peak can be seen at about 4 min, which does not record the maximum of radioactivity in blood (**Figure 3E,F**). Manual blood sampling (**Figure 3E**) and image-derived analyses (**Figure 3F**) were still possible and comparable to the correct outcomes.

[Place Figure 3 here]

FIGURE LEGENDS:

Figure 1: The two-tissue compartmental model. The physiological conditions of the three different tracer states and the dynamic processes between them are displayed.

Figure 2: Scheme of the measurement setup. (**A**) Schematic drawing of the measurement setup. (**B**) Photo of the connected shunt system with the twilite detector, peristaltic pump and

different connector types. The time-course of radioactivity in blood of a rat is detected while the animal (1) is scanned in the PET/CT (2). Therefore the arterial (a) and venous (b) catheter is connected to the detector pump system via adapter pieces (connector orange, connector blue and connector green). The arterial blood is then pumped from the arterial catheter through the detector (3) to a peristaltic pump (4) and back into the body via the venous catheter. A 3-way valve (7) is integrated in the tube system to perform tracer injection, manual blood draws and rinsing. A T-piece (8) is assembled to inject activity. The detector is connected with a computer to view, calibrate and correct the continuous blood data.

Figure 3: Representative results of continuous blood sampling compared to manual blood sampling. Typical arterial input functions derived from continuous blood sampling compared to manual blood sampling (left column) and continuous blood sampling compared to the image-derived approach (right column) are shown. Panels **A-D** demonstrate the results of correct implementation of the protocol in two different animals. Panels **E** and **F** illustrates a sub-optimal outcome of the measurement. All data shown were corrected for the cross-calibration factor and the background.

DISCUSSION:

The presented results are extracted from a larger-scale project on neuronal activity in a transgenic animal model of Huntington's disease compared to wildtype rats. Altogether 30 transgenic and wildtype rats were catheterized and manual and online blood sampling in parallel to [^{18}F]FDG-PET/CT was performed. Three AIFs of wildtype rats are shown here to demonstrate the range of possible outcomes of the protocol. The results of the complete project on changes of neuronal activity in an animal model of Huntington's disease will be published elsewhere.

The here described method enables fast and accurate continuous blood sampling in a big cohort and provides a gapless AIF for kinetic modeling of dynamic PET/CT data in small animals. An external blood circulation is generated to detect actual time activity in the blood of the animals; consequently a loss of blood is avoided. The surgical procedure is based on Jespersen et al.[8] and was modified to meet the needs for arterial blood sampling during the PET/CT measurements. The shunt system was validated by Weber et al.[9]. With the here used setup, an external blood volume of about 1.1 mL is running through the detector-pump system. A rat aged 4 month has a total blood volume of about 30 mL. The diameter of the femoral vein and artery is approximately 0.45−0.6 mm[10] and needs to be a little starched to insert the catheter used.

The AIF can also be measured via sporadic manual blood collection or be reconstructed from early time points of the PET images itself (image-derived). Both approaches were performed with the here presented data and compared to the continuous blood sampling.

In comparison to manual blood sampling, with online blood sampling a noticeable higher temporal resolution (here: 1800 data points per 30 min) becomes possible. Manual blood draws (here: 5 data points per 30 min) are limited to the blood volume present in the small animal, as

these samples are not pumped back into the circulation of the animal. Moreover, a maximum interval of 10–15 s is technically implementable and important information for kinetic modeling is missed. This can also be seen in the presented data, as a difference in the detected maximum of continuous and manual blood sampling is obvious (**Figure 3A,C,E**). With online blood sampling the detected peak was higher than with the image-derived input function of the ascending aorta[11] (**Figure 3B,D,F**). The Imaged-derived input function is restricted to the spatial resolution of PET scanners which results in partial volume effects[12] and is affected by the reconstructed time frames.

A general advantage of this continuous blood sampling procedure is that the tracer can be applied via the catheter, which is less prone to disturbance than injection via the lateral tail vein. Keep in mind that the tracer should be applied in a moderate volume to prevent the tracer from remaining in the beginning of the tube system. To ensure that no activity is remaining in the dead volume of the T-piece, it is flushed with heparinized saline solution afterwards. Moreover, the usage of an infusion pump is advised as it enables adjustment of the speed of the tracer injection and can contribute to more coordinated acquisition of the maximum radioactivity peak with manual blood sampling[13].

There are a few possible difficulties that might occur during protocol processing and can be handled by the following troubleshooting. A sub-optimal position of the catheters might lead to an incomplete execution of the protocol, therefore ensure that they are accurately fixed with the proximal suture and that the catheter is pushed 2–3 cm proximal into the vessel. In addition, fibrin adhesive can be used. Also formation of thrombi can clog the catheters. This can be handled by increasing the heparin concentration and subsequent flushing of the catheters or the tube system. Such a sub-optimal outcome due to clogging of the catheters is shown in the results, the maximum peak is missed (**Figure 3E**). Another critical point concerning animal protection and well-being is the length of the extracorporeal blood flow. It is therefore suggested to reduce the length of the tube system to a minimum.

When blood sampling is performed, three corrections of the resulting AIF have to be taken into account. First, plasma correction. Tracers equilibrate between plasma and blood cells, mainly erythrocytes. Depending on how fast these diffusion processes are, the available tracer is mainly present in plasma. For some tracers, the ratio of plasma to whole blood needs to be considered, such as more lipophilic ones. In these cases, plasma activity has to be determined. If [^{18}F]FDG is used, there is no need to centrifuge the blood to determine the plasma activity, as it equilibrates very fast between plasma and red blood cells and the availability of [^{18}F]FDG in plasma is similar to that in the whole blood. Secondly, metabolite correction. Many tracers are metabolized in whole blood and some of these metabolites are still radioactively labeled[14]. This fraction is present in the AIF but is not available for tissue uptake. For some tracers metabolites need to be determined in whole blood or plasma and the AIF needs to be corrected. Thirdly, dispersion correction. Dispersion is caused by several factors, including (a) the systematic time difference between the tracer arrival times in the tissue relative to the peripheral sampling site (delay correction) and (b) and the smearing of the shape of the AIF, as the tracer transport within the tube system is influenced by its first order lag (PT_1) kinetics. Several corrections

based on deconvolution have been proposed, mainly based on the model by Iida et al.[15], but most of them are susceptible to noise. A correction method which circumvents deconvolution and is therefore less prone to noise has been proposed by Munk et al.[16]. The necessary measurements to estimate the correction parameters have to be performed for every combination of tubing and tracer used. Dispersion correction should be done before time delay correction[17]. However, mainly fast tissue perfusion processes are affected by dispersion and it has also been shown, that for modeling of [^{18}F]FDG studies a dispersion correction is not absolutely necessary[18]. Therefore, in the presented examples the dispersion correction of the AIF has not been applied.

A proper calibration of the on-site dose calibrator and its regular quality control is a prerequisite for the type of cross calibration procedures presented here. However, if the activity administered to the animal is measured with the same dose calibrator, any deviation in accuracy will be cancelled out, provided that the deviation is constant and the complete cross calibration procedure has been followed, including nuclide-specific corrections (e.g., for varying half-life or different branching ratio). Using such a calibration procedure for harmonizing PET/CT systems used in human health care and research, an accuracy of at least 5−10% could be achieved[19,20].

The calibrated and corrected AIFs generated by successful implementation of this protocol enable quantification of PET/CT data for the characterization of animal disease models, testing of new therapy options, establishment of new tracers, and transferring of existing tracers into another species. Seemingly, continuous blood sampling in [^{18}]FDG-PET/CT in rats delivers the most reliable information for the calculation of the input in bio-kinetic modeling. By taking into account the individual metabolism, especially liver clearance, a more precise assessment of the relevant pathological or therapeutical effects is possible. With this practicable protocol, a higher efficiency of preclinical PET/CT data analysis is easily implementable.

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The authors have nothing to disclose.

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Figure 1

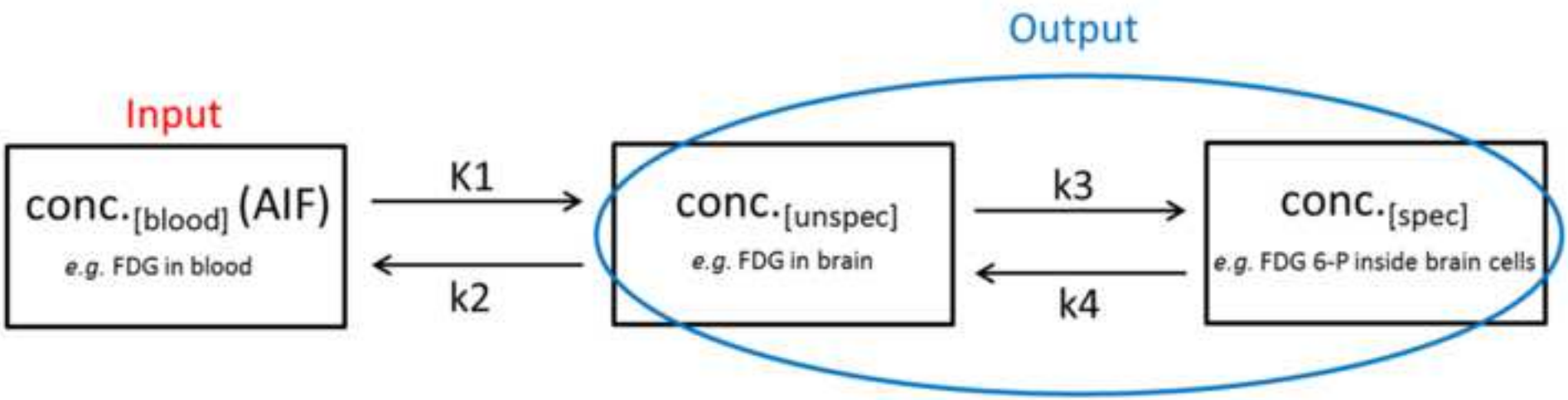

Figure 2

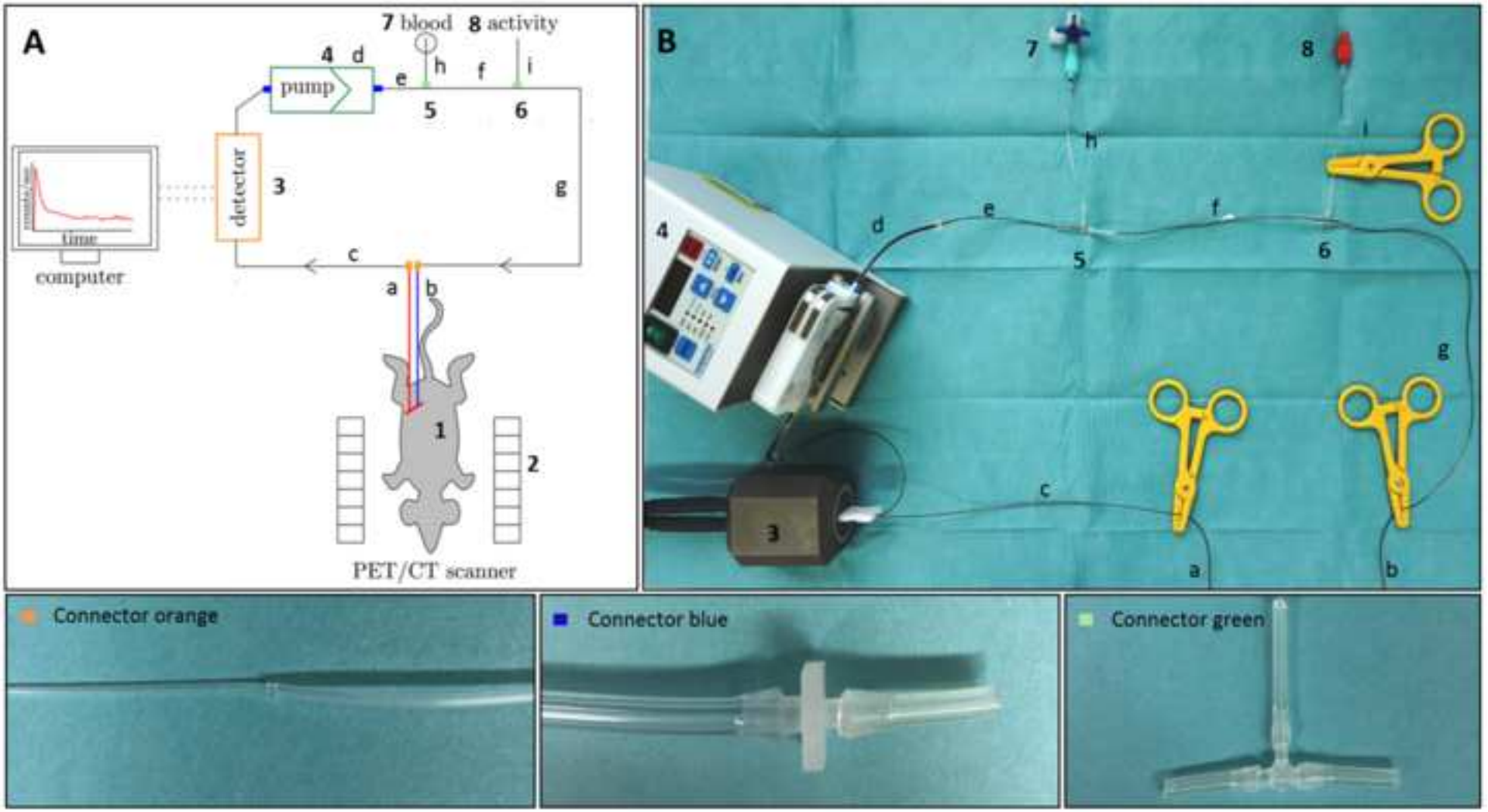

Figure 3

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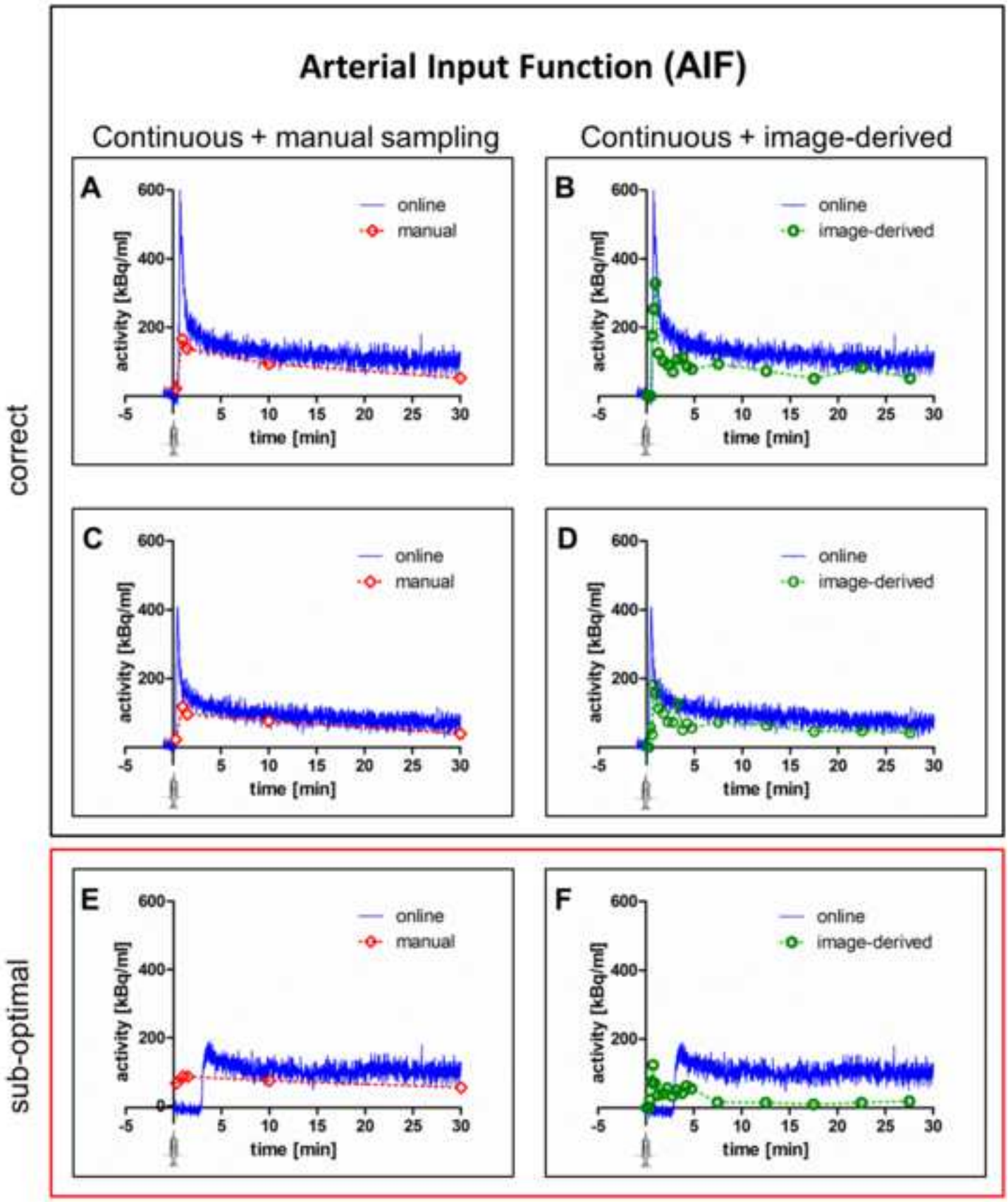

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sugery for arteriovenous shunt			
anesthesia station	Groppler		
aneurysm clips	Aesculap	FT190T	5 mm, closing force 70 g
bulldog clamp	Aesculap		35 mm
dissectiong scissors BC165	Aesculap	490-866	dull, for skin preparation
heating mat			
insulin syringe	Braun		30G
needle holder	medicon	11.62.18	micro surgical
pliers for aneurysm clips	Aesculap	FT 470T	Yasargil
portex fine bore polythene tubing	Smith Medical	800/100/200	ID 0.58 mm, OD 0.96 mm; PE50 equivalent tubing
surgical microscope with camera	Leica		M50 + MC120 HD
suture filaments 6.0			6.0, polypropylene
suture filaments 3.0			3.0, absorbable, braided
two anatomical forceps	Hammacher Soling	HSC601-11	micro surgery, 45°
vascular or corneal scissors	Geuder	G19605	micro surgery scissors
PET/CT imaging			
dose calibrator ISOMED 2010	nivia instruments GmbH		for tracer portioning
Inveon PET/CT	Siemens		
tracer (e.g. 18F-FDG)			
manuel bloodsampling			
capillary blood collection EDTA tube	KABE Labortechnik GmbH		GK 150 EDTA 200 µl
test tubes	SARSTEDT		5 ml, 75 x 12 mm, PS

well counter CAPTUS 700t	Capintec		manuel measurement of blood activity
automatic blood sampling			
BD Venflon TM pro safety shielded IV catheter; 18 G (1.3 mm x 32 mm)	BD	3932269	luer connections (to fit in t-connections)
bloodsampler twilite two	swisstrace GmbH		
combi stopper	Braun	4495101	
heparin			50U/ml for tube flushing before the experiment and aspiration during catheter surgery
hypodermic needle			G23 x 1 1/4" / 0.6 x 30 mm
microprocessor controlled tubing pump	Ismatec/Cole-Parmer	ISM596	12 rollers, 2 channels
PSAMPLE modul of PMOD	PMOD		
reduction connectors	Ismatec/Cole-Parmer	ISM569A	from ID 2.5 mm to ID 1.5 mm
silicone pump tubes	Ismatec/Cole-Parmer	070535-17-ND /SC0065N	for roller pump (yellow/blue/yellow ID 1.52 mm, WT 0.84 mm, OD 3.2 mm)
silicone pump tubes - adapter tubing	Ismatec/Cole-Parmer	SC 0107	black/black/black ID 0.76 mm, WT 0.86 mm, OD: 2.48 mm
t-piece or t-connections	Ismatec/Cole-Parmer	ISM 693A	ID 2.5 mm

ID - inner diameter; OD - outer diameter, WT - wall thickness

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Author(s): Teresa Mann, Jens Kurth, Anne Möller, Joanna Förster, Brigitte Vollmar, Bernd Joachim Krause, Andreas Wree, Jan Stenzel, Tobias Lindner

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Title: "Continuous blood sampling in Small Animal PET/CT: Measurement of the Arterial Input Function"

Corresponding Author: Teresa Mann

We thank the Editor and the reviewers for their revealing and constructive comments. We carefully discussed and commented them point by point in this document marked in blue and made necessary changes in the manuscript displayed in green (found in the reply and also in the revised manuscript). Unchanged text of the revised manuscript in this response can be found in *italics.*

Response to Editorial comments:

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

Reply: The manuscript was thoroughly proofread by the authors, both spelling and grammar errors were corrected.

2. Title: Please revise to avoid the use of punctuation (colon, dash, etc.).

Reply: Any punctuation was removed from the title. The new title is:

"Continuous Blood Sampling in Small Animal PET/CT enables the Measurement of the Arterial Input Function"

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4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Reply: Numbering of the protocol steps was adjusted to JoVE style. All dashes were removed.

5. Protocol: Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a "NOTE". Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Reply: All text in the protocol is now written in numbered steps with no more than 4 sentences. Introductory paragraphs were moved to the introduction or discussion section or broken into steps.

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Reply: We broke some of the "NOTES" into steps in imperative tense or moved them to the discussion section.

7. Line 145: Please specify surgical tools used through the protocol.

Reply: Surgical tools in line 145 were specified to:

Make an incision of about 20 mm using surgical forceps and scissors *at the groin of the rat.*

8. Line 156: How to remove leaking blood?

Reply: The removing of leaking blood in line 156 was specified in the following way:

Use corneal scissors to make a small incision into the vein (1/3 of the diameter) and remove leaking blood with a sterile cotton swap.

9. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

d) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Reply: The following information was added to the manuscript:

Place the anesthetized rat in dorsal position on a heating mat, under the surgical microscope and add vet ointment on its eyes.

e) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Reply: Does not apply. In our protocol the animals are euthanized with pentobarbital after the measurements.

f) Discuss maintenance of sterile conditions during survival surgery.

Reply: Does not apply. In our protocol the animals are euthanized with pentobarbital after the measurements.

g) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Reply: Does not apply. In our protocol the animals are euthanized with pentobarbital after the measurements.

h) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Reply: Does not apply. In our protocol the animals are euthanized with pentobarbital after the measurements.

i) Please do not highlight any steps describing anesthetization and euthanasia.

Reply: No sentences describing anesthetization or euthanasia are highlighted in the revised manuscript.

10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Reply: Shorter protocol steps were combined.

11. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

Reply: Single-line spacing was applied.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Reply: Essential steps for the production of the videos were highlighted in yellow.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Reply: No NOTES were highlighted. No sentences describing anesthetization or euthanasia are highlighted in the revised manuscript.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to

perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Reply: All relevant steps were highlighted.

15. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Reply: The Table of Materials is now sorted in alphabetical order.

Response to Reviewer 1:

1. In the discussion you stated that it is hardly possible to capture the very fast kinetic of the tracer by manual sampling. I disagree with this statement. You mention in the paper that it is possible to take a manual blood sample every 10-15 seconds. In your protocol you take the first manual sample after 30 seconds and the second sample after 60 seconds! It is pretty clear that you will miss (part of) the peak but this is not because manual sampling is not sufficient enough but because of your chosen design of the experiment/time points.
Besides that, missing the peak can also be prevented by adjusting the speed of the tracer injection. For example by using an infusion pump which is described in the literature. Based on the chosen time points and your experimental design I do not think it is reasonable to compare manual and continuous blood sampling.

Reply: These are important aspects and should be addressed in our manuscript. To overcome the limitation of the sporadic manual blood sampling within the first minute we introduced image-derived Input functions (IDIF) of the presented data sets and compare them to the continuous blood data. The time-activity information of the IDIFs was extracted from the ascending aorta. The steps how to generate the IDIF were included into the protocol:

6. Image derived Input function

6.1 Open the "Fuse it" tool on PMOD. Load the PET Image as input and the CT as reference. Click already matched.

6.2 Open the VOI tool. Place the cursor within the ascending aorta in the CT. Click predefined spherical VOI. Define a radius of exactly 0.7 mm. Extract the time activity information with the VOI statistic button and copy the averaged values to clipboard.

On the basis of the Reviewer's objection we now compare continuous blood sampling to the imaged-derived approach. The manual data is still shown in the manuscript but discussed more carefully. We also mention in the protocol that it is highly recommended to increase manual blood draws especially for early time points:

4.1 *Perform manual blood sampling 30 sec, 60 sec, 90 sec, 600 sec and 1800 sec after starting the imaging acquisition.*

Note: We highly recommend increasing the number of manual blood draws especially within the first min after tracer injection if possible. Therefore the blood sample volume has to be reduced to 20 – 30 µL per sample[1].

Moreover, we advise the usage of an infusion pump to ensure a consistent injection speed of the tracer in the discussion section. The following sentence can now be found in the manuscript:

Also, the usage of an infusion pump is advised as it enables adjustment of the speed of the tracer injection and can contribute to more coordinated acquisition of the maximum radioactivity peak with manual blood sampling[2].

2. In the introduction you mention that PET/CT allows longitudinal studies. You refer to Jespersen et al which describes a technique that makes longitudinal blood sampling possible. The group of van Waarde Mol Imaging Biol. 2016 Oct;18(5):715-23) developed a technique for repeated arterial blood sampling. It is then disappointing to see that you choose to euthanize your animals after the PET scan and not to include or mention in your protocol the possibilities of longitudinal PET studies with blood sampling.

Reply: To emphasize the possibility of longitudinal studies we included the following "NOTE" into our protocol section:

NOTE: In our study animal were euthanized after the measurements as brains were used for in-vitro analyses in our experimental design. With this setup also repeated measurements in longitudinal studies are implementable[3].

3. a An aspect which to my opinion is missing in the paper is animal welfare. The condition of the animal before, during and after the scan can have a major impact on your tracer and tracer kinetics. For example, you briefly mentioned that you keep body temperature at 37 oC by putting the animals on a heating pad. How was body temperature measured? Did you use a rectal probe or did you use the sensor on the heating pad to measure the body temperature? Please describe more clearly. If you did not use a rectal probe keep in mind that core temperate could be different from what you measured which can affect your tracer uptake.

Reply: This indeed is an important point and should be addressed in our manuscript. We measured the body temperature of the animal with a rectal probe both during the surgery and during the PET/CT scan. This information was added to the protocol.

3. b Other examples of animal welfare which are missing in the protocol are checking the condition of the animal before scanning or measuring other physiological parameters (pulse, oxygen saturation, ect) during surgery and scan. In your protocol you describe a fasting period of 12 hours to ensure stable blood glucose levels. How did you know that glucose levels are stable? Did you measure glucose levels to check and if not do you recommend this?

Reply: Physiological parameters were observed via respiration control. We did not measure blood glucose levels in this study. Though, we recommend measurement of glucose levels for every user of this protocol and addressed this in the revised manuscript:

Note: …We advise to measure glucose blood levels using manual blood draws described in point 4 to ensure stable values or to correct for in kinetic modeling.

4. Make it also very clear in your protocol that fasting is recommended for FDG scans but is not advised for every study or tracer.

Reply: To emphasize this aspect we included the following sentences into our protocol:

Note: Fasting is necessary for studies using the tracer [^{18}F]FDG but not for other tracers.

5. In the protocol you do a cross calibration between PET/CT and well counter. Is it possible and recommended to set up a comparable calibration for the dose calibrator?

Reply: According to the Reviewer's comment the following section was added to the discussion:

A proper calibration of the on-site dose calibrator and its regular quality control is a prerequisite for the type of cross calibration procedures presented here. However, if the same dose calibrator is used to measure the activity administered to the animal, any deviation in accuracy will be cancelled out, assuming that deviation is constant and the complete cross calibration procedure has been followed, including nuclide-specific corrections (*e. g.* for varying half-life or different branching ratio). Using such calibration procedure for harmonizing PET/CT systems used in human health care and research, an accuracy of at least 5-10 % could be achieved[4, 5].

Response to Reviewer 2:

1. In figure 3, can the authors also present a 3rd column of time-activity curves of the arterial input function derived from another location during PET imaging, e.g. the left atrium, as this can also be 'continuously' acquired.

Reply: The reviewer's proposal to introduce image-derived arterial input functions (IDIF) is an important aspect. We now provide IDIFs from the PET images itself in our manuscript and compare the continuous AIFs to the IDIF. The time-activity information of the IDIFs was extracted from the ascending aorta. The steps how to generate the IDIF were included into the protocol:

6. Image derived Input function

6.1 Open the "Fuse it" tool on PMOD. Load the PET Image as input and the CT as reference. Click already matched.

6.2 Open the VOI tool. Place the cursor within the ascending aorta in the CT. Click predefined spherical VOI. Define a radius of exactly 0.7 mm. Extract the time activity information with the VOI statistic button and copy the averaged values to clipboard.

2. The authors mention PET ligands are molecules involved in a metabolic pathway (Introduction, page 1, lines 73-75). This is not necessarily the case as immuno-PET radiopharmaceuticals can target cell surface proteins such as CD8 expressed by cytotoxic T-cells.

Reply: Based on the Reviewer's comment we changed lines 73-75 as follows:

Whereas the ligand is a molecule that is involved in a metabolic pathway or targets cell surface proteins,…

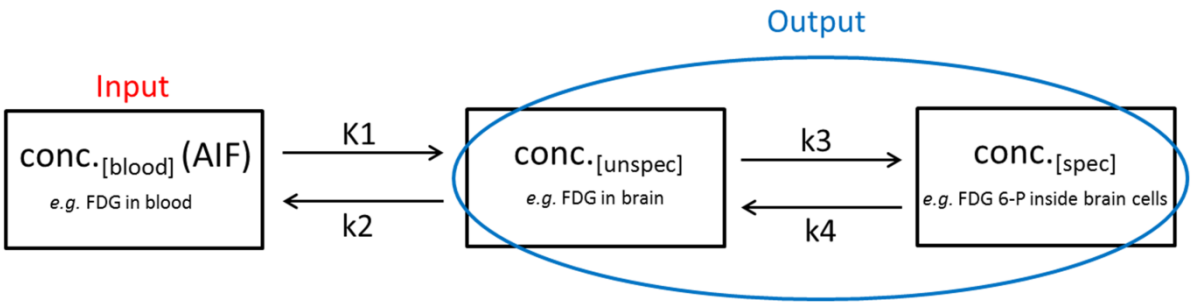
3. Page 1 line 79: 'FDG to visualize highly proliferating cell types'. This is not correct as FDG targets metabolically active cells that are not necessarily proliferating, e.g. cardiomyocytes.

Reply: Based on the Reviewer's comment we changed line 79 as follows:

…metabolic processes like glycolysis…

4. Page 1 line 86, can the authors give an example of compartmental modeling with labeled k1, k2 etc. to further characterize the dynamic nature of the process in a given tissue of interest.

Reply: We emphasized the dynamic nature of the distribution processes between the different compartments by introducing rate constants. Moreover we added a tracer example in Figure 1:



We also included the following section into the introduction:

All these dynamic processes between the compartments are to some extent bidirectional and the diffusion processes are described by rate constants (K1, k2, k3, k4).

5. Page 3: can the authors please share the expected approximate diameter of a 4-month old rat femoral artery and vein.

Reply: According to the remark of the reviewer the following information was inserted into the discussion of the manuscript:

The diameter of the femoral vein and artery is approximately 0.45-0.6 mm[6].

6. Page 3, line 153 (point 9): specify the recommended distance from the suture.

Reply: We added the following information to line 153/point 9:

1.5 *… Block the vein with an aneurysm clamp proximal, but* 2-3 mm *distal from the suture with the bulldog clamp.*

7. Page 5 - image acquisition: are any measures (in addition to suturing) taken to avoid the catheters from being dislodged as the animal is placed into the micro-PET scanner.

Reply: No other measures in addition to suturing have been taken. In our experiments the careful handling of the animals was sufficient. No catheters were dislodged. Nevertheless, in our manuscript we note in the discussion section that fibrin adhesive can be used in addition to fix the catheters in the right position and prevent them from shifting.

8. Can the authors add the approximate volume of blood in a 4-month old rat, and the appr. volume that circulates through the pump + catheters at a given time.

Reply: To provide better insights to this issue for the readership we introduced the following sentence into the discussion section:

With the here used setup an external blood volume of about 1.1 mL is running through the detector-pump system. A rat aged 4 month has a total blood volume of about 30 mL.

9. Have the authors experienced ventricular tachyarrhythmias with their method and / or hemodynamic instability?

Reply: We did not experience ventricular tachyarrhythmias or hemodynamic instability. Animals were stable during the complete PET/CT measurement and normally breathing.

10. How is the peristaltic pump rinsed / disinfected between animals for re-usage?

Reply: The tube system was only used for the measurement of one animal. After the measurement the complete tube system was replaced. The pump itself is disinfected regularly. To clarify this aspect to our readership we inserted the following sentence into our protocol:

…Use a completely new tube system for the next animal.

11. Figure 2A: the figure is pixelated. Can a higher resolution figure be inserted. The text between h and f is not legible (? 3-way valve).

Reply: We inserted a higher resolution figure in 2A and corrected the labeling.

References:

1. Napieczynska, H. *et al.* Impact of the Arterial Input Function Recording Method on Kinetic Parameters in Small-Animal PET. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. **59** (7), 1159–1164, doi: 10.2967/jnumed.117.204164 (2018).

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3. Sijbesma, J.W.A. *et al.* Novel Approach to Repeated Arterial Blood Sampling in Small Animal PET: Application in a Test-Retest Study with the Adenosine A1 Receptor Ligand [(11)C]MPDX. *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging*. **18** (5), 715–23, doi: 10.1007/s11307-016-0954-9 (2016).

4. Geworski, L., Knoop, B.O., de Wit, M., Ivancević, V., Bares, R., Munz, D.L. Multicenter comparison of calibration and cross calibration of PET scanners. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. **43** (5), 635–9 (2002).

5. Boellaard, R. Standards for PET image acquisition and quantitative data analysis. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. **50 Suppl 1**, 11S–20S, doi: 10.2967/jnumed.108.057182 (2009).

6. Liu, H.-L. Microvascular anastomosis of submillimeter vessels-a training model in rats. *Journal of hand and microsurgery*. **5** (1), 14–7, doi: 10.1007/s12593-013-0089-z (2013).