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Standardization of small animal imaging : current status and future prospects

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Standardization of small animal imaging - current status and future prospects

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Author Comments:	Dear Dr. Gibson, please find enclosed our manuscript entitled "Standardization of small animal imaging - current status and future prospects" that we submit to Molecular Imaging and Biology as review article. Co-authors of the paper are Firat Kara, Janine Doorduin, Kerstin Fuchs, Gerald Reischl, Sayuan Liang, Marleen Verhoye, Felix Gremse, Laura Mezzanotte and Marc C. Huisman. Corresponding author is J. G. Mannheim, contact information as follows: Dr. Julia Mannheim Department of Preclinical Imaging and Radiopharmacy University of Tuebingen Roentgenweg 13 72076 Tuebingen, Germany Phone: +49-7071-2982974 Fax: +49-7071-2925133 E-mail: julia.mannheim@med.uni-tuebingen.de Non-invasive in vivo small animal imaging has evolved from a niche research application into a powerful and scientifically significant tool for basic research. Small animal imaging enables faster translation and application of preclinical insights into the clinical routine and thus plays a pivotal role in biomedical and pharmaceutical research. However, the overall benefit of small animal imaging and the insights obtained, e.g., in specific diseases, are directly linked to the validity and reliability of the collected data. If the data (regardless of the modality used) are not reproducible and/or reliable, then the outcome of the data is rather questionable. Therefore, standardization of small animal imaging is of paramount importance. In this review, we will address the current status of standardization in preclinical imaging, as well as potential benefits from increased levels of standardization. A separate chapter focuses on animal handling in general, followed by sequential chapters focusing on the imaging modalities PET, MRI and OI. The modalities CT and SPECT are not addressed. Potential areas for standardization will be listed for each modality, and the current status as well as future prospects will be discussed. We hope that our review is suitable for publication in Molecular Imaging and Biology. All authors agree with the submitted version of the manuscript. Please do not hesitate to contact me in case of questions. Kind Regards, Julia Mannheim
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Standardization of small animal imaging - current status and future prospects

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Abstract

Non-invasive *in vivo* small animal imaging has evolved from a niche research application into a powerful and scientifically significant tool for basic research. Small animal imaging enables faster translation and application of preclinical insights into the clinical routine and thus plays a pivotal role in biomedical and pharmaceutical research. However, the overall benefit of small animal imaging and the insights obtained, e.g., in specific diseases, are directly linked to the validity and reliability of the collected data. If the data (regardless of the modality used) are not reproducible and/or reliable, then the outcome of the data is rather questionable. Therefore, standardization of small animal imaging is of paramount importance. When PET systems from different vendors are employed for individual studies, possible comparisons are hampered by the differences in, e.g., acquisition parameters (such as energy or timing window, etc.) that can differ from vendor to vendor. Each vendor might offer different reconstruction algorithms, different protocols for normalization and calibration of the system and different output formats. These factors must all be considered when standardization across sites and different scanner vendors is desired. It may be preferable to characterize the resulting quantitative accuracy of the image data than to standardize every individual step in the image generation process. With the exception of animal preparation/injection, quantitative accuracy and image quality parameters can be standardized via a process similar to that used to develop the European Association of Nuclear Medicine Research Ltd. (EARL) accreditation program. In a second step, the influence of animal preparation/injection on the reproducibility of the imaging data can be assessed by measurements on a small number of animals using the acquisition and reconstruction protocols obtained in step 1. In this review, we will address the current status of standardization in preclinical imaging, as well as

potential benefits from increased levels of standardization. A separate chapter focuses on animal handling in general, followed by sequential chapters focusing on the imaging modalities PET, MRI and OI. The modalities CT and SPECT are not addressed. Potential areas for standardization will be listed for each modality, and the current status as well as future prospects will be discussed.

Keywords: small animal imaging, standardization, reproducibility, reliability, PET, MRI, OI, animal handling

1. Introduction

Non-invasive *in vivo* small animal imaging has evolved from a niche research application into a powerful and scientifically significant tool for basic research [1]. Small animal imaging enables faster translation and application of preclinical insights into the clinical routine and thus plays a pivotal role in biomedical and pharmaceutical research. Small animal imaging has many key benefits over conventional methods (e.g., invasive endpoint studies): protocols and results can easily be translated to clinical studies; longitudinal measurements can be performed on the same animal, diminishing inter-animal variability and consequently increasing statistical significance; one imaging session can provide a multiplicity of molecular and functional parameters; and the number of animals can be dramatically reduced [2]. A multitude of different imaging modalities are available; each has pros and cons and should be chosen depending on the study design and scientific goal.

While positron emission tomography (PET) and single photon emission computed tomography (SPECT) provide a variety of biological targets for investigation of functional and metabolic pathways [1, 3-6], magnetic resonance imaging (MRI) offers clear delineation of organs due to its high soft tissue contrast, as well as functional parameters, e.g., apparent diffusion coefficients (ADCs), for investigating diffusion in a specific tissue [7]. Optical imaging (OI), either based on the detection of fluorescence, chemiluminescence or bioluminescence, can be applied to image certain reporter genes. However, planar imaging approaches are hampered by the lack of quantification and the low tissue penetration depth, which may be overcome by three-dimensional methods such as fluorescence-mediated tomography (FMT) [8]. By contrast, computed tomography (CT) provides high-resolution bone imaging but lacks soft-tissue contrast and exposes the patient/animal to a certain radiation dose [9-10].

However, the overall benefit of small animal imaging and the insights obtained, e.g., in specific diseases, are directly linked to the validity and reliability of the collected data. If the

data (regardless of the modality used) are not reproducible and/or reliable, then the outcome of the data is rather questionable. Many results cannot be compared, occasionally even within one institution, because they depend on different complex factors such as anesthesia, animal handling, physiological parameters, data acquisition, and analysis. These factors can greatly influence the outcome of experiments, but most are in general controllable (e.g., acquisition parameters) and thus to a certain degree avoidable. Standardization across sites in a multicenter approach, however, is more difficult to achieve since more factors are involved, e.g., different scanner manufacturers, site specificity of scan protocols, tracer production on-site vs. delivery (which is directly linked to the amount of injectable specific activity) and personnel training. To overcome these differences, standardization of protocols and procedures as well as determination of the quantitative accuracy of different vendor systems are strongly required both within one institution but also in multicenter approaches.

Standardization of protocols and parameters has been extensively performed in the clinical setup for different scanner modalities, for patient preparation in general and for different imaging agents [11-15]. The best example may be the role of harmonization of image quality in clinical [^{18}F]Fluorodeoxyglucose ([^{18}F]FDG) PET, with a prominent role of European Association of Nuclear Medicine (EANM) guidelines for PET/CT tumor imaging. Based on this work, response monitoring criteria were enlarged and complemented (as envisioned by the progression from "Response Evaluation Criteria in Solid Tumors" (RECIST) to "PET Response Criteria in Solid Tumors" (PERCIST)) [16]. Comparability of results, a field standard of working with standard operation procedures (SOPs), and complete and transparent reporting of the results obtained would prevent the need for duplicate studies and consequently contribute to refinement and reduction, and, finally, to a greater return-of-cost in the preclinical environment [17-19]. However, apart from the rationale for standardization, it is also important to examine the practicability of standardization. Some of the employed parameters can easily be standardized (e.g., acquisition parameters, such as energy or timing

window for PET, echo time (TE) or repetition time (TR) for MRI, and voltage or current for CT)), but other parameters are more demanding to standardize, e.g., certain aspects of animal handling (animal facility, personnel training), cross-calibration of different vendor systems or image analysis in general (experience of analysis).

2. Standardization of small animal imaging

In this review, we will address the current status of standardization in preclinical imaging, as well as potential benefits from increased levels of standardization. A separate chapter focuses on animal handling in general, followed by sequential chapters focusing on the imaging modalities PET, MRI and OI. The modalities CT and SPECT are not addressed. Potential areas for standardization will be listed for each modality, and the current status as well as future prospects will be discussed.

2.1 Animal handling in general

The field of small animal imaging in preclinical research has expanded in the last few years due to its high potential to analyze functional, anatomical and physiological processes non-invasively in living animals in follow-up studies over a long time period (depending on the animal model and the imaging methodology). Several factors, such as anesthesia, animal handling, fasting, and administration of the imaging agents, can influence the outcome and reproducibility of each study. To reduce the number of animal studies and to achieve high reproducibility and international comparability among multiple research groups, imaging protocols must be standardized. Animal handling plays a major role and has a great influence on the outcome of quantitative data.

Potential areas of standardization include the following:

1) Age, weight, animal strains, and various aspects of housing conditions
2) Anesthesia and animal monitoring: general
3) Anesthesia in small animal PET and MRI

2.1.1 Age, weight, animal strains, and various aspects of housing conditions

A logical first step in the standardization of imaging studies is the use of rodents of the same strain, age and animal weight within and between studies with a similar research question, as there are marked functional and behavioral differences between strains, ages and weights [20-21]. It is also necessary to standardize the vendor and not use rodents from different vendors within one study or multiple studies in the same animal model [20-23]. In addition, when performing multi-center studies in rodents, for example, studies on standardization of imaging, rodents should all be ordered from the same vendor. Transportation stress also has an impact on the animal physiology and should be considered during standardization of imaging experiments [22-25]. The content of the diet should be investigated before initiating a longitudinal study and should be considered as a source of variation when the results of different research centers are compared using the same animal model. However, there are also reports suggesting that environmental standardization may give rise to idiosyncratic results [26-28]. Richter et al. suggested that environmental standardization instead of heterogenization may cause poor reproducibility of experimental outcomes [26]. By contrast, van der Staay and colleagues emphasized the importance of standardization, and they suggested that standardization is inevitable for the risk assessment of new therapeutic drugs and prohibits random variation [29].

In most biomedical research, particular in the field of neurology, including studies involving imaging, only male rats are used due to differences in the developing brain and a 10% larger total brain size in male rats compared to female rats [30]. For other studies, females are often preferred due to their compatibility with each other, which allows female animals to be placed together in cages. However, a recent meta-analysis supported the use of both male and female rodents, by demonstrating that the variability between females was not greater than that observed in males and that females could be included to limit generalization of findings [31]. Given these known gender differences, males and females should not be mixed in a single experiment unless it has been demonstrated that it will not affect the outcome of the study.

How rodents are housed affects their welfare and hence the way they cope with stressful experimental handling. Differences and changes in housing conditions can therefore have a large effect on the experimental outcome of imaging studies when this is not taken into account, especially in neuroimaging studies.

Guidelines exist for the cage size and the number of rodents housed in a single cage, although several studies have challenged these recommendations [32-33]. While group size itself can have an effect on rodent welfare, an even larger effect can be observed when rodents are housed singly. Another important aspect of rodent housing is the use of environmental enrichment to improve living conditions by meeting the need for rodents to, for example, make nests, find shelter and gnaw, and will positively affect welfare [34]. However, whether environmental enrichment would increase the variability between rodents and thus negatively affect standardization has been questioned [34]. It is thus important to find a balance between enrichment for improving rodent welfare and avoiding the introduction of variability.

Rodents can identify human experimenters by smell, and Sorge *et al.* was the first to demonstrate that the presence of humans (either male or female) can affect the study outcome [35]. For example, male experimenters caused reduced pain behavior in mice compared with female experimenters, suggesting that standardization of animal handling should include the sex of the experimenters within one laboratory, especially in stress-related studies [35].

2.1.2 Anesthesia and animal monitoring: general

In imaging experiments, the use of anesthesia can often not be avoided as rodents must be constantly restrained. Different anesthetic agents have different effects on rodent physiology and, consequently, the study outcome [36]. Moreover, anesthetics have different targets and therefore have different effects on brain function during the period that the rodent is anesthetized. While anesthetics differentially affect physiology, they all cause a significant reduction of the rodent's body temperature. This reduction itself can affect the physiology of

the rodent. Especially in imaging studies in which changes in blood flow can affect the outcome of the study, such as functional MRI studies and studies in which tracers are injected, this decrease in body temperature should be taken into account.

When performing imaging studies, the same anesthesia should always be used and should be standardized as much as possible. Although anesthesia can, in general, not be avoided, the body temperature of the rodents during scans should be maintained at a normal and constant level using, e.g., heating pads.

To standardize anesthesia and temperature within an experiment, it is important to monitor physiological parameters. For example, during the scan it is possible to monitor the heart rate, breathing, blood oxygen levels and temperature. Keeping these parameters stable over time and as similar as possible for each rodent by adjusting or standardizing the amount of anesthetics can avoid variation in the imaging outcome.

2.1.3 Anesthesia in small animal PET and MRI

In terms of a possible impact of the anesthesia used, Fuchs *et al*. analyzed pCO_2, pH and lactate values in mice before and after [^{18}F]fluorothymidine ([^{18}F]FLT) PET investigations with different breathing and anesthesia protocols in an inflammation (arthritic) and cancer (colon carcinoma) mouse model. Significant changes in pCO_2 and lactate values were observed in anesthetized compared to conscious mice breathing air or oxygen [37]. This effect was mainly caused by sustained respiratory acidosis due to oxygen breathing, which caused increased pCO_2 and reduced lactate and pH values in rodents and thus affected the results of the study. Interestingly, a significant increase in uptake was observed in the muscle tissue used as control tissue in colon carcinoma-bearing mice under anesthesia compared to awake mice. Since muscle tissue is often used as a reference uptake region compared with tumor uptake, these results should be considered when analyzing the acquired data [37].

Mahling et al. focused on tumor hypoxia imaging using [^{18}F]fluoroazomycin arabinoside ([^{18}F]FAZA) and the effect of the anesthetics used (isoflurane vs. ketamine/xylazine while breathing air or oxygen) on tracer uptake [38]. Higher tumor uptake was observed in ketamine/xylazine-breathing mice (for both air and oxygen), and lower whole-body uptake was observed when isoflurane was used for anesthesia, clearly revealing that anesthesia substantially influences the tracer uptake in PET imaging under hypoxic conditions [38].

Isoflurane, ketamine/xylazine, medetomidine/midazolam and pentobarbital are frequently used as anesthetics in preclinical studies. Different anesthetics can have different effects on mouse physiology, such as glucose metabolism, heart functions, blood pressure and breathing frequency [37]. Additionally, mice anesthetized with ketamine/xylazine show increased serum glucose levels [39], whereas decreased glucose utilization is observed in rat brains [40]. Further, xylazine alone, which stimulates the α_2-adrenergic receptor on pancreatic islands, causes hyperglycemia in mice evaluated using [^{18}F]FDG [41]. The effects of ketamine alone on cerebral glucose utilization can be reversed by administration in combination with xylazine in specific regions [40]. Volatile anesthetics such as isoflurane lead to open mitochondrial ATP-regulated potassium channels, whereas propofol or pentobarbital have no effect on these channels [42].

The impact of anesthesia on reproducibility has been investigated extensively for a variety of different PET tracers (e.g., [^{18}F]FDG, [^{18}F]FLT or[^{18}F]FAZA), but studies have been limited for other tracers, e.g., C-11-labeled substances, such as [^{11}C]raclopride for D2-receptor imaging, [^{11}C]-3-amino-4-(2-dimethylaminomethylphenylsulfanyl)-benzonitrile ([^{11}C]DASB) for serotonin-receptor imaging or [^{11}C]Pittsburgh compound B ([^{11}C]PIB) to determine amyloid deposits in Alzheimer’s disease. Especially in the preclinical setting, these tracers, among others, are used extensively for various models [43-45], and hence the imaging routines for these tracers must be standardized to obtain reliable and highly diagnostic results.

Different studies have examined possible effects of different anesthetic regimes on structural, functional and/or pharmacological MRI [46-51] and magnetic resonance spectroscopy (MRS) studies [52]. Anesthetic regimes and doses should be carefully selected, particularly for functional MRI and MRS studies. While isoflurane is the most commonly used anesthetic for structural imaging due to the fast recovery of exposed animals, a variety of anesthetic regimes are used for functional and/or pharmacological MRI, including α-chloralose, medetomidine, propofol and urethane. The pros and cons of these anesthetics have been comprehensively discussed in earlier reviews [46-51]. Due to the side effects of anesthetics on functional connectivity, cerebral hemodynamics and brain metabolism, awake imaging of rodents has been attempted for imaging studies [53]. Although awake imaging of mice and/or rats may overcome the disadvantages of anesthetic regimes for physiological parameters, more studies are needed to standardize the methods used for awake imaging using a variety of MR techniques.

2.2. PET

Non-invasive *in vivo* PET is a powerful tool enabling the investigation of molecular, metabolic and functional parameters due to the variety of available specific radiopharmaceuticals.

Preclinical PET is a powerful tool for basic research that facilitates the assessment of molecular and functional processes of diseases as well as potential therapies due to the multitude of available animal disease models [5, 54]. A variety of small animal PET scanners have been developed by either university institutes or companies [55-57]. This development has led to an increase in the number of preclinical PET research studies. Many of these studies have been based on qualitative/visual interpretation of images. However, the demand for quantitatively accurate data is increasing due to 1) the increasing role of preclinical data in obtaining approval for new drugs and 2) the increase in the power of clinical PET due to harmonization for, e.g., response monitoring as well as studies that cannot be performed in a single-center setting (due to, e.g., patient inclusion criteria).

Potential areas of standardization include the following:

1) Animal preparation (particularly issues such as fasting, warming, glucose)
2) Quality assurance in tracer production
3) Scanner quality control, acquisition parameters & image reconstruction
4) Data analysis (including post processing of image data)

2.2.1 Animal preparation

The scientific validity of a small animal PET study is greatly dependent on the standardization of biological aspects that can influence the acquired data [58]. Several studies have shown that animal preparation, tracer injection or uptake can greatly influence the PET uptake and, consequently, reproducibility.

Fueger et al. investigated the effect of ambient temperature, anesthesia and dietary state on the biodistribution of [^{18}F]FDG in mouse tumor models [39]. Figure 1 depicts examples of the biodistribution from Fueger et al. [39]. A profound influence of these parameters on the tumor visualization and the biodistribution of [^{18}F]FDG was detected [39]. These results have been confirmed by others [59-60].

The diet of the animals and the duration of fasting before imaging can also affect the study outcome, particularly for PET studies using the glucose analog [^{18}F]FDG. Overnight fasting of mice results in a decreased level of plasma glucose and an increased concentration of [^{18}F]FDG in plasma [61]. Brain [^{18}F]FDG uptake was higher in mice after fasting than in non-fasting mice, whereas myocardial uptake was lower [61-62]. Thus, the effects of fasting on [^{18}F]FDG are apparently tissue dependent.

For brain research, higher [^{18}F]FDG uptake would lead to better image quality and image analysis, and thus fasting is preferred over non-fasting. Deleye *et al.* studied mice under fasting durations of 0 to 24 hours [63]. The authors concluded that a fasting duration of at least 12 hours should be considered to obtain reproducible brain [^{18}F]FDG uptake. When using a shorter fasting duration, brain [^{18}F]FDG uptake should be corrected for plasma glucose to obtain reproducible data [63]. The optimal fasting duration for rats has not been determined but is likely comparable to that in mice.

To reduce variation in [^{18}F]FDG uptake, fasting of animals should be considered. Most importantly, the fasting duration should be standardized within a study and between studies when data must be compared.

2.2.2 Quality assurance in tracer production

Tracer production of radiopharmaceuticals for clinical use is conducted under stringent quality control (QC) procedures following good manufacturing practice (GMP) principles. In addition to screening for volume, activity, activity concentration and specific activity, testing

for QC involves detailed verification of identity (e.g., half-life and gamma spectrometry), testing for chemical purity (using high-performance liquid chromatography (HPLC) and gas chromatography (GC) especially for residual solvents, pH-value), microbial purity (endotoxins, sterility), radionuclidic purity (short or long lived impurities) and radiochemical purity (HPLC and/or thin layer chromatography (TLC) for known and unknown impurities). Additionally, a reference sample from each production batch must be stored for at least one year to enable subsequent analysis if needed.

In the preclinical setup, in sharp contrast to the clinical setup, the quality control of the produced tracer is not subject to GMP procedures. However, most of the above-mentioned verification tests are performed, especially for well-established preclinical tracers and in well-established radiopharmacies. However, especially for radiotracer development, in which new substances are labeled and *in vitro* and *in vivo* tests are first performed, most of the quality control tests will not be executed, and these tests will only be performed if the developed tracer shows promising results and is scheduled for a full, in-depth *in vivo* evaluation. This circumstance should be considered in standardizing small animal imaging.

2.2.3 Scanner quality control, scan acquisition parameters & reconstruction

The reproducibility of acquired PET data can be greatly affected by a variety of technical parameters, such as the reconstruction algorithm, data analysis, software version or applied corrections. Some of these parameters and their impact on PET data have been examined in detail [64-65]; although both of these studies focused on clinical parameters, the results should be easily translatable to preclinical studies.

For absolute quantification, multiple corrections of PET data are needed. Attenuation and scatter correction might be difficult to standardize depending on the acquisition type (external source vs. CT). Unfortunately, even within one institution, standardization of technical aspects can be difficult because they depend on various complex factors such as data

acquisition, reconstruction and analysis. Comparison of the acquired data might not be feasible for longitudinal studies during which the software from the scanner's vendor is upgraded. However, standardization of technical aspects across different sites is even harder to achieve and ultimately might not be fully possible. When PET systems from different vendors are employed for individual studies, possible comparisons are hampered by the differences in, e.g., acquisition parameters (such as energy or timing window, etc.) that can differ from vendor to vendor [57]. Each vendor might offer different reconstruction algorithms, different protocols for normalization and calibration of the system and different output formats [57]. These factors must all be considered when standardization across sites and different scanner vendors is desired.

It may be preferable to characterize the resulting quantitative accuracy of the image data than to standardize every individual step in the image generation process. With the exception of animal preparation/injection, quantitative accuracy and image quality parameters can be standardized via a process similar to that used to develop the European Association of Nuclear Medicine Research Ltd. (EARL) accreditation program. In a second step, the influence of animal preparation/injection on the reproducibility of the imaging data can be assessed by measurements on a small number of animals using the acquisition and reconstruction protocols obtained in step 1.

2.2.4 Data analysis

The contribution of data analysis to reproducibility can also be assessed. Data analysis in PET can be highly demanding, particularly for kinetic modeling or multiparametric image studies. Standardization of data analysis might be limited by differences in software as well as the expertise of the operator analyzing the data, which can impact the reproducibility and reliability of the acquired data. Therefore, each operator should be trained accordingly using specific training datasets with known outcomes to ensure the reproducibility of image

analysis. However, there is no common standard for such training and is currently addressed on individual basis by each institution. Multiple analysis software solutions that fit the different needs of individual studies are available, ranging from proprietary developments to fully licensed software solutions by scanner manufacturers or third-party companies. The reliability and reproducibility of these software packages has not been investigated in detail. The validity of in-house software developed for data analysis should be carefully evaluated and compared with fully licensed software to ensure reliability (using basic parameters for comparison such as %injected dose (ID)/cc (%ID/cc) or standardized uptake values (SUVs)). Finally, guidelines for reporting of PET small animal experiments have been suggested, and we strongly advise adhering to these guidelines [66].

2.3. MRI

Magnetic resonance techniques (i.e., magnetic resonance imaging and spectroscopy) are excellent non-invasive imaging tools that can map a wide range of tissue parameters [67-68] or, for example, provide information about the concentration of brain metabolites that are less abundant than water in the brain [69].

Preclinical MRI and MRS techniques have been increasingly used to perform longitudinal studies to obtain neuro-imaging fingerprints of subtle changes in animal models for neurodegenerative, psychiatric and other central nervous system-related disorders, such as stroke & cancer [70]. In contrast to clinical MRI & MRS, preclinical MR techniques require administration of anesthetics (to prevent motion), which can interfere with the MRI results (see chapter 2.1.3). In addition, variations in animal housing conditions, handling, strain differences and MR techniques, hardware and related software might affect inter- and intra-study variability. One of the limitations of pre- and clinical MR techniques is the lack of consensus on standardized and optimized MRI and MRS methods. Studies have increasingly focused on improving clinical system standardization for multiple MR techniques and establishing multicenter platforms for central nervous system disorders [71-81]. Furthermore, clinical MRI accreditation programs and manuals for evaluating MRI performance are available (see www.acr.org/accreditation). We are not aware of such accreditation programs for standardization of preclinical MR techniques in single-center and/or multicenter research platforms.

Potential areas of standardization include the following:

1) Animal preparation
2) Scanner QC, acquisition parameters & image reconstruction
3) Image analysis
4) Introduction of field standard protocols for common imaging tasks

2.3.1 Animal preparation

The subject-specific sources of variation include diversity in experimental set-up, including animal handling, positioning of the animal in the scanner and fixation [82]. Variations in anesthetic regimes, route of administration, physiological parameters, gender, strain, circadian cycles and diet can affect the results of animal MRI/MRS experiments. A detailed explanation of the physiological effects of anesthetic drugs is beyond the scope of this review, but this information can be found in the literature [83]. To increase data quality and decrease inter- and intra-subject variability, the physiological parameters of the animals (e.g., respiration rate, heart rate, body temperature, blood pH, blood oxygenation, CO_2, blood pressure and body weight), and environmental conditions (transport, temperature, diet, microenvironment, light-dark cycle of the animalarium, cage enrichment) should be adequately monitored and controlled. Great attention should be paid to uniformly positioning the animal with respect to the RF coil(s), as this affects the loading of the coil, which is related to coil sensitivity (see [82] for a detailed discussion). The outcomes of a variety of MRI techniques, such as resting state functional MRI (fMRI) networks [84-85], neuroanatomy [86] and cerebral metabolite levels [87] are dependent on the rodent strain. Less studied causes of variation in MRI experiments are animal stress (which alters corticosteroid levels) and diet. The impacts of different stress models on MRI have been depicted [88-90]. Acclimation of the animals to the scanner room and handling may decrease the physiological stress levels of the animals. Depending on the experimental context, the type of diet may play an important role in functional and structural alterations of brain networks [91]. When the conditions mentioned above are not controlled, variations among experiments performed using MR techniques may occur. The experimental set-up of experiments and physiological monitoring should be standardized to minimize subject-related variations.

2.3.2 Scanner QC, acquisition parameters & image reconstruction

The reproducibility of the acquired MRI data is hampered by scanner quality, scan acquisition (and reconstruction) parameters (and steps), and software versions or applied corrections. Scanner quality control steps provide more insight about instrument-related sources of variations in MRI experiments that may hamper the experimental reproducibility. Two main instrument-related sources of variations in MRI experiments are hardware-related differences (e.g., field strength, RF coils, RF amplifier, gradients, shim unit, and signal-to-noise ratio) and hardware imperfections, such as magnetic field drift, scanner noise, partial volume effects, eddy current effects, static field inhomogeneity, differences in magnetic susceptibility, image intensity inhomogeneity, imperfect gradient amplifier calibration and gradient coil non-linearity [92-93]. Preclinical studies performed at ultra-high magnetic field strengths may be particularly influenced by different hardware imperfections compared with studies performed at lower fields. All types of MR techniques (Table 1) are sensitive to differences in image acquisition via sequence parameter settings such as echo time, repetition time, flip angle, number of slices, slice orientation, direction phase encoding, acquisition volume, number of averages, and microenvironment (i.e., scanning environment and temperature) [71, 94-95]. Controlling such variations is important for preclinical MRI standardization. These different influences can be identified and controlled by implementing scanner quality assurance programs based on dedicated phantoms (e.g., scanner performance stability) [95-97]. In preparing this review, we contacted preclinical research centers and found that quality control protocols using phantoms were generally not applied.

As discussed in an earlier chapter, standardization of technical aspects within an institution or between different research centers can be difficult because of the involvement of different complex steps, such as data acquisition, reconstruction and analysis. For multicenter studies, a consensus on which data acquisition, reconstruction and analysis methods should be employed, can be established. If this is not possible, all datasets should at least pass

predefined quality control assessments to reduce the risk of obtaining divergent results due to problems in image quality, including artifacts.

Two approaches have been suggested to reduce variability due to scanner quality, acquisition parameters and image reconstruction methods within and between different labs [71]. First, the use of the same equipment, scanners, and software for preclinical studies will eventually decrease the variability between studies carried out in the same MRI research center and between other centers for multicenter studies. However, it is difficult to enforce the use of the same technology because MRI-related technology is continuously improving. If inter-scanner variance is considered noise, the acquired data can be modified before analysis, or alternatively, scanner affects can be adjusted statistically as described elsewhere in more detail [71]. The Function Biomedical Informatics Research Network (FBIRN) project and others have suggested the development of methods to measure and/or decrease scanner-associated variations using dedicated phantoms [71, 95-96, 98-100]. A protocol for FBRIN phantoms and the processing software are publicly available through their website [100]. Phantom measurements as described by the FBRIN website and other sources, can assess many important quality characteristics, such as the signal-to-noise ratio, signal-to-fluctuations noise ratio (measure temporal stability), signal drift, image uniformity, ghosting artifacts, chemical shift and spatial resolution, slice thickness accuracy, slice position accuracy, and low-contrast object detectability etc. [97, 100-102]. We conduct routine stability tests (as suggested by FBRIN website) in our institute (Bio-Imaging lab, Antwerp University) using a phantom to check instrument-related variations. Our routine stability tests help us detect subtle fluctuations related to the coil and/or MRI instrument as well as image quality. FBRIN and other websites (http://www.acr.org) provide MRI quality manuals that can be used for routine tests. In these documents, weekly assessment of quality control is suggested. There is a need for online platforms where users can compare their quality control results with each other. Such comparisons may aid the detection of hardware- or software-related performance

changes. Additionally, the standardization and use of phantom quality programs in each preclinical lab may enable the creation of platforms for multicenter studies.

The FBIRN approach seems to be similar to the concepts of cross-calibration and image quality used within PET, in which phantom image characteristics are standardized, instead of every possible setting, during scanner QC, data acquisition and image reconstruction.

2.3.3 Image analysis

Standardization of data analysis methods for MR data is hampered by, among other factors, the availability of multiple data analysis techniques and software (and updates). There is a need for systematic analysis of all data analysis methods to compare the efficiency of these methods with each other. For standardization purposes, a sample dataset that has passed through predefined quality control assessments can be used to compare results from different data analysis techniques as well as software. The same dataset should also be analyzed by more than one person using the same data analysis methods to estimate whether the data analysis steps are adequately standardized and reproducible.

2.3.4 Introduction of field standard protocols for common imaging tasks

Clearly defined protocols are required to decrease instrument- and software-related errors/differences, maximize data quality and increase the reproducibility of results obtained by independent researchers. Instead of standardizing each individual parameter during image reconstruction, it would be useful to define a dedicated set of specifications for a given imaging application and ask centers to ensure, through adjustments of the individual parameters, that the end result of their imaging procedure is compliant with those specifications.

An essential component of standardization is identifying what and how to standardize. It is therefore important to have insight on the sources of artifacts and pitfalls of the specific MR

techniques. Brief information about different MR techniques commonly employed in preclinical imaging can be found in Table 1. MR techniques are grouped according to their application fields, namely, functional, structural or metabolic. Table 2 provides references to guidelines for tips, tricks and pitfalls of a variety of MR techniques and critical elements of data processing.

2.4. OI

Optical imaging is defined as imaging techniques and methods that rely on the detection of fluorescence and bio/chemiluminescence [103-105].

2.4.1 Fluorescence

In fluorescence reflectance imaging (FRI), excitation light interacts with fluorochromes inside the mouse, causing re-emission of photons, which are captured by a camera. FMT is a similar technique that involves more sophisticated hardware but allows tomographic three-dimensional reconstruction of the fluorescence (Fig. 2). FMT corrects for different depths and heterogeneities of absorption and scattering, ameliorating or even resolving the limitations of planar reflectance imaging [106]. FMT is frequently combined with micro-CT (μCT), which provides anatomical information valuable for improved image reconstruction and analysis [107-108].

Potential areas of standardization include the following:

1) Phantom-based calibration of fluorescent probes
2) Animal preparation
3) Multimodal FMT imaging protocol
4) Image analysis

2.4.1.1 Phantom-based calibration of fluorescent probes

Phantoms are important to assess the brightness and stability of fluorescent probes or to assess the image quality of novel devices or reconstruction methods. They can be constructed using silicon rubber or gelatin in combination with substances for scattering, absorption and fluorescence, such as titanium oxide powder, lipid emulsions, India ink and fluorescent dyes

[109-110]. Alternatively, a plastic phantom of size 15x33x40 mm with diffuse optical properties resembling average mouse tissue can be ordered from a hardware supplier [105]. This phantom contains a cylindrical inclusion for 100 µl of the substance under investigation. A small amount (4%) of lipid emulsion should be added to ensure that the optical scattering of the inclusion resembles the rest of the phantom [108]. For many applications, a reproducible and stable reference dye is required [109-110], and a set of calibrated dyes at multiple wavelengths can be obtained from PerkinElmer, the manufacturer of a commonly used FMT system [108, 111].

2.4.1.2 Animal preparation

While usage of well-defined phantoms is justified and important, the *in vivo* situation is much more complicated due to irregularities in shape, strong heterogeneities with respect to absorption and scattering, and the presence of motion due to breathing and heart beating. Furthermore, probes and particles may behave differently than anticipated in blood or other tissues due to, e.g., the presence of enzymes and opsonizing proteins.

For fluorescence imaging, nude mice bear significant advantages because the removal of hair from normal hairy mice can cause strong infectious reactions and irritation. Furthermore, in some mouse strains (e.g., C57BL/6), pigmented regions frequently remain, which affect the imaging in an unpredictable manner. Furthermore, nude mouse strains differ in size, e.g., BALB/c nude mice are typically smaller than CD1 nude mice; both strains are frequently used for tumor experiments due to their immune deficiencies. Small mice bear advantages for FMT because resolution and sensitivity are reduced in deep tissue regions [109]. Immuno-competent nude mice are also available, e.g., SKH1-mice or Black six nude mice. Additionally, the use of µCT contrast agents may affect the optical imaging. For example, AuroVist, a long-circulating agent, shows strong optical absorption, which is apparent as

purple skin color [112]. Diet, i.e., type of chow, may also seriously affect fluorescence experiments by increasing the background signal, particularly for wavelengths below 750 nm, which can be avoided by using a special chlorophyll-free chow [113-114].

2.4.1.3 Multimodal FMT imaging protocol

Standardization of an FMT imaging protocol requires setting many parameters, such as the wavelength, number and distance of laser injection points, field of view or sensitivity parameters that determine the exposure time. Such a protocol was described in detail for µCT-FMT imaging using two commercially available devices and a multimodal mouse holder [108]. The protocol involves advanced fluorescence reconstruction using heterogeneous absorption and scattering maps and has been applied in several studies [109, 114-118]. While this protocol is specific for a special FMT device, replacement of the µCT is possible. Figure 2 shows fused CT and FMT images acquired using this standardized protocol. The mouse was prepared with a rectal insertion containing fluorescence and CT contrast agent to enable assessment of the fluorescence reconstruction quality. Such a rectal insertion is a compromise between a phantom and an *in vivo* experiment with an intravenously injected probe, thus providing a balance between realism and complexity, and was recently used to assess the sensitivity and accuracy of FMT in deep tissue regions [114].

2.3.1.4 Image analysis

Image analysis is another critical step in imaging studies that may benefit from standardization. The availability of anatomical µCT data allows reproducible segmentation of organs, lesions and tumors, which reduces the inter-reader variability compared with unimodal FMT usage [107, 119]. While some organs such as the kidney and bladder are easy to segment, the liver is more difficult due its lobular structure, resulting in higher variability between users [107]. Therefore, fully automated organ segmentation may become a valuable

tool, particularly for biodistribution studies [120]. The rectal insertion shown in Figure 2 can be segmented in a user-independent way by means of thresholding and region growing. Therefore, such an approach can be valuable to assess and compare image quality between devices or reconstruction algorithms in a robust and standardized way. Finally, digital curation is becoming increasingly important, and standardized formats to curate multimodal volumetric image data and even entire imaging studies would be desirable [121-122]. Accordingly, the development and utilization of such protocols are desirable to assess and improve the accuracy and robustness of CT-FMT studies.

2.4.2. Bioluminescence

In vivo bioluminescence imaging (BLI) is performed as follows. A luciferin substrate is administered to the animals prior to acquisition of light signals and their processing using dedicated low-light imaging systems such as CCD cameras. The real advantage of BLI is the exquisite sensitivity and specificity of the technique at the molecular level and the high signal-to-background ratio of the bioluminescent reaction. These advantages are particularly notable when using firefly luciferase with D-luciferin [123]. Bioluminescence tomography (BLT) allows the generation of a 3D reconstruction of signals for more precise localization of signals. BLT imaging data can be co-registered with CT imaging data for more precise localization of signals *in vivo* [124].

Potential areas of standardization include the following:

1) Methods to assess the brightness and stability of bioluminescent probes or standards probes
2) *In vivo* imaging
3) Image analysis

In this scenario, many elements can be subject to standardization: devices, software version, probe and substrate injection route and dose in animals, protocols for BLI measurement (including scan settings), mouse models, reconstruction settings for BLT, and data analysis.

Efforts to standardize any of these parameters have been limited. One reason is the difficulty of identifying an appropriate standard probe. For bioluminescence, the use of purified enzymes as standards presents many challenges, whereas for fluorescence standardization, molecules with a defined quantum yield can be used. In fact, for the majority of applications luciferase reporters are usually expressed in cells, and absolute quantification is unachievable because expression varies with time and cell conditions. Interestingly, a standardized reference imaging protocol seems to have emerged, as evidenced by the high number of papers reporting this protocol in their materials and methods [125-127].

This particular BLI protocol images firefly luciferase-expressing cells in anesthetized nude mice 10 minutes after intraperitoneal injection with a dose of 150 mg/kg of D-luciferin. As a starting point for future work, it would be interesting to assess the precision and robustness of cell imaging using a luciferase-expressing cell line in different laboratories. First, the robustness of cell lines *in vitro* can be evaluated as a standard. Second, the magnitude of the influence of imaging parameters can be evaluated using the imaging conditions described above.

Although this standard protocol is good for many applications (e.g., imaging of subcutaneous tumors in mice), it has serious limitations for other applications. For example, a dose of 150 mg/kg D-luciferin does not saturate firefly luciferase in many organs such as the brain, and intraperitoneal injection might not the best route of injection. For brain applications, a higher dose of substrates injected intravenously guarantees higher sensitivity [128-129]. Moreover, application of the same protocols to cells expressing low levels of luciferase can fail to generate detectable signals.

3. Discussion & Outlook

In this review, the current status of standardization for various preclinical imaging modalities (PET, MRI and OI) is presented. The level of standardization varies considerably, which is most apparent from the different contributions of phantom experiments in practice (far more in PET than in OI). By contrast, the trend toward standardized image acquisition protocol in OI is in line with the clinical EARL FDG PET protocol, which suggests acquisition settings and a set of phantom experiments to confirm that the obtained image quality parameters are within an acceptable range, allowing for multi-center studies. In general, this methodology appears to be a good strategy for PET and MRI by focusing on QC issues, acquisition hardware, and image reconstruction and providing a higher threshold for multi-centric reproducibility of small animal imaging studies. The standardized imaging protocol could subsequently be applied to animals, probably leading to lower observed reproducibility due to animal preparation-induced variability. In theory, a dedicated imaging protocol permits the determination of baseline values of reproducibility for the preclinical parameter of interest, which could inform, e.g., statistical analysis of the number of animals needed to test a certain hypothesis.

In short, the utility of preclinical imaging would definitely be enhanced by improved standardization. Approaches do exist for the implementation of the next steps in quantification, and it is encouraging that practical initiatives for their realization are currently underway in the context of European Society of Molecular Imaging (ESMI) and EANM workgroups. This would be a natural follow-up to the important work on 'Guidance for methods descriptions used in preclinical imaging' [66], which was intended to ensure that each report on a small animal imaging experiment contains the essential information required to understand and reproduce the experimental work.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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FIGURE 1: Impact of animal handling on the biodistribution of [^{18}F]FDG. (A) Not fasted, warmed, no anesthesia. (B) Fasted, not warmed, no anesthesia. (C) Fasted, warmed, no anesthesia. (D) Fasted, warmed, no anesthesia, conscious injection. (E) Reference conditions: not fasted, not warmed, no anesthesia. (F) MicroCT, sagittal view for anatomic reference. (G) Not fasted, warmed, isoflurane. (H) Fasted, warmed, isoflurane. (I) Fasted, warmed, ketamine. This research was originally published in *JNM*. From Fueger BJ, Czernin J, Hildebrandt I, et al. (2006) Impact of animal handling on the results of 18F-FDG PET studies in mice. *J Nucl Med.* 2006 Jun;47(6):999-1006. © by the Society of Nuclear Medicine and Molecular Imaging, Inc.

FIGURE 2: Fluorescence imaging. A nude mouse (BALB/c nu/nu) was anaesthetized, prepared with a rectal insertion containing a known amount of fluorescence, and imaged with µCT-FMT. A) The reflectance image acquired by the 2D mode of FMT shows the mouse. B) The fluorescence image is shown as a color-coded overlay, and the rectal insertion appears as a diffuse hyperintense region, which complicates analysis. C) The multimodal mouse bed holds the mouse between two transparent acrylic glass plates (green). Markers (red) are used for automated fusion. The segmentation of the mouse body (orange) is used for fluorescence reconstruction. D) The reconstructed 3D fluorescence distribution (shown as an overlay at the bottom) appears at the rectal inclusion. The inclusion can be identified in the µCT data due to the addition of µCT contrast agent. Hence, this approach can be used to assess the image quality of the fluorescence reconstruction in a reproducible manner. E) 3D rendering of the µCT-FMT data showing the co-localization of the fluorescence with the insertion. This example shows that standardization of fluorescence imaging involves various aspects, including mouse models, animal preparation, probe design, imaging devices, scanning protocols, and image analysis.

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TABLE 1: Summary of preclinical MRI and MRS techniques

Application	Imaging types	Main characteristics	*References*
Functional	Task-dependent fMRI, phMRI, rsfMRI, MEMRI, Perfusion	Functional MRI (fMRI) techniques investigate stimuli (e.g., visual) driven local neural signal changes by observing alterations in cerebral blood flow and blood oxygenation concentrations (i.e. blood oxygenation level dependent, or BOLD contrast). This is an indirect way of measuring neural activity since the amplitude of these local signal changes depends on a variety of factors such as cerebral metabolic rate of oxygen consumption, cerebral flow/volume and neuronal activity. Pharmacological drugs can be employed in order to investigate hemodynamic changes in the brain using pharmacological MRI (phMRI). In contrast to task dependent fMRI, the course of stimulus in phMRI depends on the pharmacological agent. Resting state fMRI provides connectivity maps by evaluating the correlation between spontaneous low-frequency (<0.1 Hz) BOLD fluctuations between functionally related areas during rest. Manganese-enhanced MRI (MEMRI) is considered a valuable tool in preclinical MRI for monitoring neuronal activity (thanks to the paramagnetic property of Mn2+ which acts as a Ca2+ analog to enter excitable cells through Ca2+ channels). The perfusion MRI studies the blood flow in the brain tissue in response to pathology and metabolic needs.	[130-139]
Structural/ Anatomical	T_1-, $T_2^{(*)}$-, PD-weighted imaging, DWI, DTI, MEMRI. FLAIR, MTI, Relaxometry (T_1 and T_2), MRA	Structural MRI provided quantitative and qualitative information based on biophysical status of the tissue. In contrast to functional MRI, which is sensitive to temporal changes in neuronal activity, structural MRI is more sensitive to biophysical changes in the tissue which can be detected by transverse relaxation time ($T_2^{(*)}$), longitudinal relaxation time (T_1) and proton density (PD) weighted images. Diffusion weighted images (DWI) can be also employed to investigate changes in white and gray matter integrity. Diffusion tensor imaging (DTI) can investigate axonal networks within white and gray matter. It provides essential information about structural connectivity within central nervous system. MEMRI allows visualization of anterograde connections (e.g., olfactory pathway) and enhance the visualization of cytoarchitecture of the brain. Fluid attenuated inversion recovery technique (FLAIR), which is highly T_2 weighted, is used collect images where cerebral spinal fluid (CSF) is nulled which allows better visualization of lesions near CSF. Magnetization transfer imaging (MTI) is being used main to investigate white matter alterations in the brain. Iron content of the tissue can be detected with susceptibility-weighted imaging (SWI). T_1 and T_2 relaxometry (i.e. relaxation rate), acquired with MRI, provide quantitative information about the bio-physical environment of the tissue in health and disease. Magnetic resonance angiography (MRA) provides information about the anatomy of cerebrovasculature.	[140-147]
Metabolic	MRS	Magnetic resonance spectroscopy (MRS) plays an important role in determining the concentration of different metabolites non-invasively based on their chemical shift in certain areas of the brain.	[69, 148-149]

Abbreviations: MRI: magnetic resonance imaging; fMRI: functional MRI; phMRI; pharmacological MRI; rsfMRI: resting state fMRI; MEMRI: Manganese-enhanced MRI; T_2: transverse relaxation time; T_2^*: T_2 star; T_1: longitudinal relaxation time; PD: proton density; DWI: Diffusion weighted images; DTI: Diffusion tensor imaging; FLAIR: fluid attenuated inversion recovery technique; MTI: Magnetization transfer imaging; MRA; Magnetic resonance angiography; MRS: Magnetic resonance spectroscopy.

TABLE 2: References for pitfalls, artifacts and technical considerations MR techniques

Application	Imaging types	References for pitfalls, artifacts and technical considerations
Functional	fMRI	[67, 135, 150-160]
	MEMRI	[161-162]
Structural/ Anatomical	DTI	[157, 163-166]
	Relaxometry (T1 and T2),	[167-171]
	MRA	[172-175]
Metabolic	MRS	[149, 175-177]

Abbreviations: MRI: magnetic resonance imaging; fMRI: functional MRI; MEMRI: Manganese-enhanced MRI; DTI: Diffusion tensor imaging;T2: transverse relaxation time; T2*: T2 star; T1: longitudinal relaxation time; PD: proton density; MRA; Magnetic resonance angiography; MRS: Magnetic resonance spectroscopy

Figure

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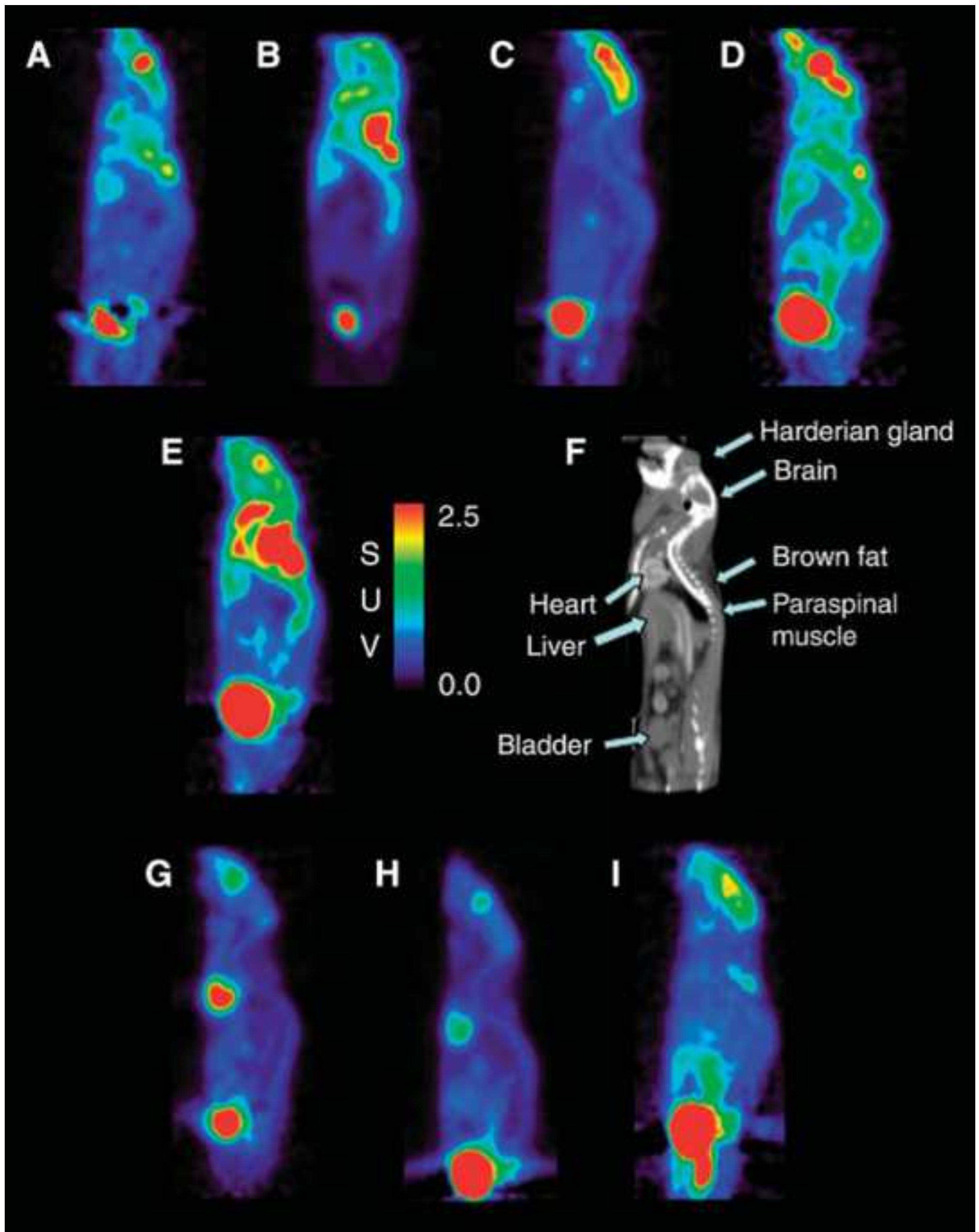

Figure

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