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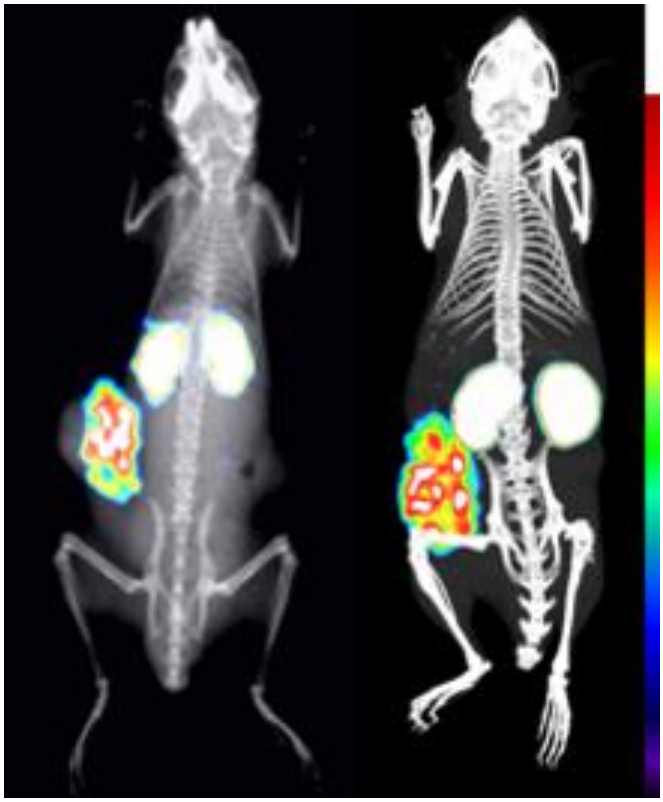

WHITE PAPER

Quantification in 2D, 3D imaging and *ex vivo* biodistributions for preclinical oncology studies; strengths and limitations.

Maritina Rouchota†[1], Panagiotis Tsialios*[1], Irineos Pilatis[1], Benjamin Larimer[2], Chanelle Hunter[2], Suzanne Lapi[2], Hailey Houson[2], Adriana Massicano[2], Anna Sorace[2], Theodosia Maina[3], Berthold A. Nock[3], Panagiotis Kanellopoulos[3], Sophia Sarpaki[1], Maria Georgiou[1], Eleftherios Fysikopoulos[1], George Loudos[1]

[1] BIOEMTECH, 27 Neapoleos str., Lefkippos Attica Technology Park - NCSR "Demokritos", 15310 Athens, Greece
[2] Small Animal Imaging Facility – Department of Radiology, The University of Alabama at Birmingham, USA
[3] Molecular Radiopharmacy, INRASTES, NCSR "Demokritos", 15310 Athens, Greece

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1. Introduction

Over the last decade, dedicated small-animal imaging systems have been continuously evolving and developing in technology, mostly driven by the demand for efficient drug development and the desired fast translation from the cellular level to the clinical level (1). Applications in small-animal imaging are strongly motivated by preclinical *in vivo* validation of drug effectiveness and safety, especially in nuclear imaging, such as single photon emission tomography (SPECT) and positron emission tomography (PET) imaging. Preclinical imaging using site-directed radionuclide carriers can both demonstrate the interaction of the tumor with its microenvironment and exploit functionality to better define the tumor volumes or characterize specific tumor sites (2). Moreover, preclinical applications help to validate the dedicated animal models themselves, by assessing the expression of different drug receptors in the desired organs or induced pathology models under study (1). For these reasons, preclinical studies hold the most prominent tool in oncology research, driving the progression of new drugs towards clinical use.
While the evolution in preclinical imaging is quite significant in recent years, the gold standard in any preclinical oncology experiment is still *ex vivo* biodistributions, an invasive method that requires a large number of animals to be used (3). However, *ex vivo* biodistributions use metric scales that are, in some cases, more consistent between animals and humans (4, 5).

Already published studies have shown that both planar (2D) and tomographic (3D) imaging provide a high correlation to *ex vivo* studies (6, 7). As an example, a detailed study showed that radiopharmaceutical uptake evaluated in removed tumors, is associated with that resulting from *in vivo* planar ($r = 0.94$, $P < .05$, $n = 18$) and SPECT ($r = 0.90$, $P < .05$, $n = 18$) images. The percentage of injected dose per gram of excised tumors from biodistribution measurements, is highly correlated with the same measure derived from planar ($r = 0.90$, $P < .05$, $n = 18$) or SPECT ($r = 0.87$, $P < .05$, $n = 18$) images (8). These results are also reinforced by the results of a recent study, focused on combining planar and tomographic imaging to provide optimal preclinical results, using a smaller number of animals per study (9).
From a quantification point of view, these models also provide the drug exposure-response relationship that is essential for understanding the degree of antitumor activity linked to the drug under study. This allows the *in vivo* interpretation of tumor growth inhibition data that is later used for early clinical development (10).
In the present study, a set of 29 different oncology imaging studies, performed both on 2D and 3D commercial systems, are analyzed and compared. Results are compared against the gold standard biodistribution data, to evaluate the correlation between the three different methods of targeting quantification in preclinical oncology studies. In addition, the ability of the 2D imaging technique to provide a faster and more cost-effective approach is investigated, as this approach combines various advantages including whole body image information, multiple time points on the same animal, dramatic minimization of required animals and significant reduction of overall study time and associated costs.

2. Materials and Methods

2.1 Imaging Systems

Real-time, fast, dynamic screening studies were performed on dedicated benchtop, mouse-sized, planar scintigraphy systems (γ-*eye*™ by BIOEMTECH, Athens, Greece for SPECT isotopes detection and β-*eye*™ by BIOEMTECH, Athens, Greece for PET isotopes detection). Both systems support fusion with a digital mouse photograph, for anatomical co-registration extraction of the X-ray mouse image, which is extracted from the mouse photo using artificial intelligence tools and can be used for mapping purposes (11).

Tomographic SPECT/CT imaging was performed with two commercial tomographic systems; more specifically, γ-CUBETM and x-CUBETM (Molecubes, Belgium) and GNEXT PET/CT (Sofie, USA). The γ-CUBETM (Molecubes, Belgium) system provides SPECT images (12) and the accompanying x-CUBETM (Molecubes, Belgium) can provide CT images (13). The nuclear imaging components do not include attenuation correction, but the CT component is calibrated periodically to read HUs.

2.2 Animal studies

Mouse imaging on tomographic systems, was performed by keeping the mice anesthetized under isoflurane and under constant temperature of 37°. SPECT scans were acquired with a 30 min – 2 hours duration, based on the injected activity and system specifications. Each SPECT scan was followed by a CT scan for co-registration purposes. The SPECT data for the Molecubes system were reconstructed through an MLEM algorithm, with 250 um voxel size and 500 iterations. Images were decay corrected and normalized between administration routes. CT data were reconstructed through an ISRA algorithm, with 100 um voxel size. All PET data on the GNEXT system, are collected in list mode format allowing frame durations (temporal acquisition) to be determined after acquisition allowing for flexibility during post-processing and uses 3D-OSEM reconstruction algorithm.

For the current study, a total of 29 different oncology studies, were deployed. All animal studies that have been considered, originate from licensed protocol procedures that have already been published, according to international standards and are referenced here, in the relevant analysis. A total of 29 animals, which originated from different study protocols: different mouse strains, different tumor models and different targeting tracers, were the basis of this study. Preclinical oncology mouse models analyzed across a variety of radiopharmaceuticals and divided into imaging comparisons and biological validation. More specifically, a set of 18 oncology studies (i.e., 18 selected animals, coming from different study designs, different animal tumor models and tracers) were analyzed and compared for the agreement between 2D and 3D quantification results. A set of 11 oncology studies (i.e., 11 selected animals, coming from different study designs, different animal tumor models and tracers) were analyzed and compared for the agreement between 3D imaging and *ex vivo* biodistribution quantification results and a set of 5 oncology studies (i.e., 5 selected animals, coming from different study designs, different animal tumor models and tracers) were analyzed and compared for the agreement between 2D imaging and *ex vivo* biodistribution results.

For SPECT isotopes, studies with Tc-99m and In-111 labeled compounds were analyzed. For both cases, different peptides targeting the tumor were used. More information and details on selected peptides that have been included in this analysis, can be found in recent publications (14, 15, 16, 17, 18). For PET imaging, Co-55 and Ga-68 labeled compounds were used. Again, the goal of these studies was tumor targeting in different oncology models.

In all study cases, bolus intravenous injections of tumor-targeting agents were performed, with compound volumes between 100 – 150 uL. Mice were kept anesthetized during administrations and during imaging with isoflurane anaesthesia (induction with 3-5% isoflurane flow rate and maintenance with 1-3% flow rate). In half of these studies (when no longitudinal scanning was needed) mice were euthanized right before imaging, to stop the compound kinetics. In the cases where animals were imaged alive, studies started with 2D imaging, and the 3D scans followed right after. For the comparison with biodistribution data, animals were imaged and euthanized right after the scans were completed. Tissues were extracted and stored until the radioactivity values were compatible with the gamma counter measurements.

All the *in vivo* experiments that provided the basis of this analysis, have been published, with each protocol described in detail, in the relevant publication. More analytically, (i) for In-111 labeled compounds the oncology studies that were analyzed for comparison purposes, are presented in (14) and (16), (ii) for Tc-99m labeled compounds, the studies deployed are presented in (15), (17) and (18). For (iii) the PET studies, the details of the oncology studies with Ga-68 and Co-55, can be found in (11).

The protocols and all the animal procedures were approved by the General Directorate of Veterinary Services (Athens, Attica Prefecture, Greece) and by the Bioethical Committee of BIOEMTECH Laboratories (Permit number: EL 25 BIOexp 045) based on the European Directive 2010/63/EU on the protection of animals used for experimental purposes. For the experiments performed at UAB, all experiments were licensed under the IACUC 20317 (Institutional Animal Care and Use Committee) of the institution. In addition, all animal procedures were performed by highly trained personnel with certifications in animal handling.

2.3 Quantification from imaging

For the live dynamic imaging performed with γ-*eye*™ and β-*eye*™, post-processing and quantification is performed through the embedded analysis software, visual| *eyes*. Regions of interest (ROIs) are drawn on major organs of interest, the tumor and around the whole mouse. ROIs are drawn with anatomical guidance from the built-in system's anatomy imaging component (i.e., mouse photograph or x-ray image, according to the user's choice). The count rate per ROI is immediately shown on the post processing of the embedded software and after a simple division with the whole mouse counts, the %counts/organ is easily and quickly extracted. This analysis was used for the comparison with 3D imaging. The formula used for both 2D and 3D imaging is the following:

$$\text{Image \% counts / organ} = (\text{organ counts from image}) / (\text{whole body counts from image}) * 100$$

For the comparison with *ex vivo* biodistributions, extra steps were added to extract the results in %ID/organ values.
The Calibration procedure, included in the visual|*eyes* software, as previously described (5), was exploited. Conversion factors transforming counts/min to activity are implemented for each isotope, allowing an immediate activity measurement of each ROI right by the end of the acquisition. By simply dividing this value with the measured injected activity (injection syringe on dose calibrator), the %ID/organ is extracted.
For the tomographic images acquired with the different tomographic imaging systems (both SPECT and PET), post-processing and quantification were performed through third-part analysis software, VivoQuant v1.23 (Invicro LLC, Boston). Volumes of Interest (VOIs) are drawn on major organs of interest and then divided by the number of counts of the whole mouse. This provides the %counts/organ with respect to the animal counts.
Again, for the comparison with *ex vivo* biodistributions, certain extra steps were added to extract results in %ID/organ values. This time, animals were measured in a dose calibrator, while being anaesthetized, right before imaging and the counts measured in the image were translated into actual activity, for each animal. Based on this translation, the %counts/organ measured for each organ from the image and the injected activity (injection syringe on dose calibrator), the %ID/organ is extracted. The following formula is used, based on the exported conversion factors:

Image % ID / organ = (organ activity from image) / (injected activity from dose calibrator) * 100

2.4 Quantification via post-imaging *ex vivo* biodistributions

Mice from the relevant studies were dissected and organs of interest (e.g., kidneys) and tumors were collected, weighted, and counted in a gamma counter (automated well-type gamma counter Canberra Packard Auto-Gamma 5000 series). In view of the high radioactivity doses injected into animals as required for imaging, samples were kept in the freezer for a certain time period to reach lower radioactivity levels and ensure reliable measurements within the linearity zone of the counter. Biodistribution data were calculated as percent of injected dose per organ (%ID/organ) with the aid of suitable standards of the injected dose.

BDs % ID / organ = (organ activity from gamma counter) / (injected activity from gamma counter)

2.5 Statistical analysis

For statistical comparison between 2D/3D, 3D/ *ex vivo* and 2D/ *ex vivo* biodistribution results, independent samples t-tests were conducted. The level of statistical significance of the t-tests was set at $p < 0.05$. All statistical analyses were performed using the GraphPad Prism 9 (GraphPad Prism Software, San Diego, CA).

3 Results

3.1 Comparison between 2D and 3D imaging

The comparison between 2D and 3D imaging quantification data was implemented on 18 different oncology studies, as already described. The %counts/organ value was used as the value of comparison, for both SPECT and PET isotopes.
The overall difference of %counts/organ values between 2D and 3D imaging is seen in **Table 1**:

Mean difference between 3D/ *ex vivo* biodistributions (%)	**10.80**
SD (%)	**11.74**

Table 1. *The mean difference and the standard deviation (%) calculated on 18 different oncology studies, on the same animals imaged on 2D and 3D imaging (n=18).*

Indicative imaging results of the 2D/3D imaging comparison, both for Tc-99m and In-111 agents are shown in depicted in **Figure 1**:

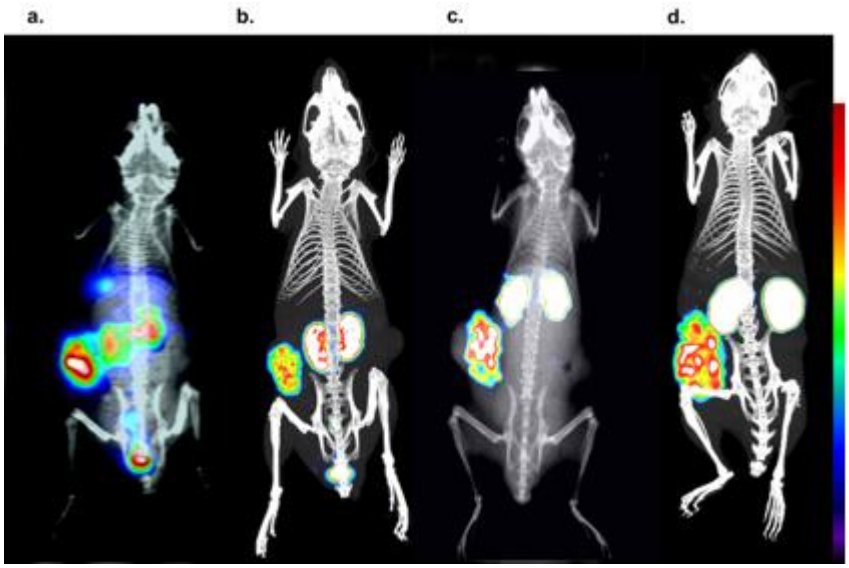

Figure 1 ***Tc-99m labelled tumor targeting peptide imaged with 2D and 3D imaging respectively and c, d) an In-111 labelled tumor targeting peptide imaged with 2D and 3D imaging, respectively. The color bar indicated the accumulation level (i.e., white being the highest and blue the lowest).***

Indicative imaging results of the 2D/3D imaging comparison, for Co-55 and Ga-68 compounds are presented in **Figure 2**:

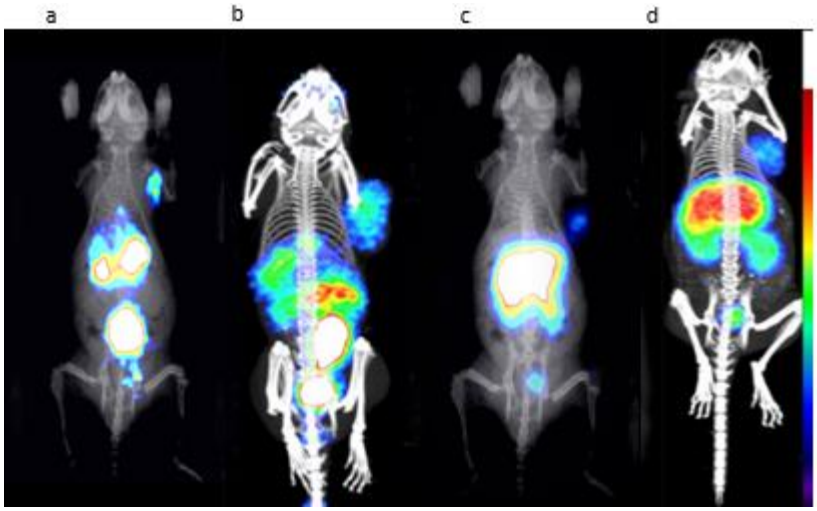

Figure 2 ***a, b) A 68Ga-GZP targeting peptide, imaged with 2D and 3D imaging respectively and c, d) a 55Co-labeled SSTR2 targeting peptide imaged with 2D and 3D imaging, respectively. The color bar indicates the accumulation level (i.e., white being the highest and blue the lowest).***

3.2 Comparison between 3D and *ex vivo* biodistributions

The comparison between 3D imaging and *ex vivo* biodistributions quantification was implemented on 11 different oncology studies, as already described. The %ID/organ value was used as the value of comparison.

The overall difference of %ID/organ values between 3D imaging and *ex vivo* biodistributions, is seen in **Table 2:**

Mean difference between 3D/ *ex vivo* biodistributions (%)	**22.02**
SD (%)	**11.15**

Table 2. *The mean difference and the standard deviation (%) calculated on 11 different oncology studies, on all the animals imaged on 3D imaging and studied through ex vivo biodistributions (n=11).*

3.3 Comparison between 2D and *ex vivo* biodistributions

The comparison between 2D imaging and *ex vivo* biodistributions quantification was implemented on 5 different oncology studies, as already described. The %ID/organ value was used as the value of comparison.

The overall difference of %ID/organ values between 2D imaging and *ex vivo* biodistributions, is seen in **Table 3:**

Mean difference between 2D/ *ex vivo* biodistributions (%)	**18.00**
SD (%)	**17.35**

Table 3. *The mean difference and the standard deviation (%) calculated on 5 different oncology studies, on the same animals imaged on 2D and studied through ex vivo biodistributions (n=5).*

3.4 Statistical analysis

The conducted t-tests revealed no statistically significant differences among the three different uptake quantification techniques. The afore mentioned results are summarized in both **Table 4** and **Figure 3**.

Uptake quantification techniques' comparison	Sample size (n of animals)	Statistics
2D vs 3D	18	t = 0.752; p = 0.453
3D vs *ex vivo* biodistributions	11	t = 0.087; p = 0.931
2D/*ex vivo* biodistributions	5	t = 0.513; p = 0.627

Table 4. *The overall statistical difference between techniques which are under comparison. No statistically significant differences were observed. The statistical significance level was set at p = 0.05.*

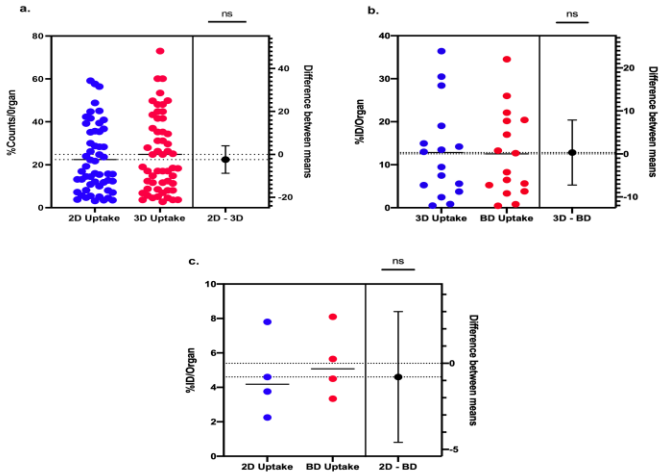

Figure 3 ***The schematic overview of the results of the techniques' statistical comparison via estimation plots. Each estimation plot shows the data (%ID/Organ) as a scatter graph (left axis) and the precision of the calculated effect size as a 95% confidence interval (right axis). a. Comparison between 2D and 3D imaging uptake quantification studies, b. Comparison between 3D imaging uptake quantification and ex vivo biodistribution uptake quantification studies and c. Comparison between 2D imaging uptake quantification and ex vivo biodistribution uptake quantification studies. When the 95% confidence interval***

includes zero the p-value is greater than 0.05 and the statistical difference is not significant. Consequently, these three estimation plots confirm the results of Table 4 revealing no statistically significant differences among the three different uptake quantification techniques.

4 Discussion

The evolution in preclinical imaging is quite significant in the recent years, providing a very strong tool for evaluating new oncology drugs for tumor targeting and identifying novel predictive imaging biomarkers of therapeutic response. Even if imaging gains an important role in preclinical oncology research, *ex vivo* biodistributions still hold a prominent role, often regarded as a gold-standard of quantification in drug targeting efficacy evaluation. This is primarily due to both the high cost and complicated imaging analysis required to extract this information. Especially for radiopharmaceutical development, statistics (differences across animals), allometry (differences between animals-patients, but also between clinical and small-animal cameras) often lead to unreliable estimations when based only on one method. Each of the two methodologies should go hand-in-hand during new drug evaluation.

The three methods compared in the present study, seem to provide similar results with no significant statistical difference ($p = 0.45 – 0.93$), over the chosen oncology studies. The deviations that have been noticed can be a result of various parameters. An important factor that can cause deviations in the results, is the exact time point of study in longitudinal studies. Since kinetics in a living animal cannot be avoided, by scanning the same animal with 2D imaging and immediately after on 3D imaging, will inevitably have small or bigger differences in organ distribution of the tracer, depending on the compound kinetics. This becomes evident by the absolute activity measurements of the animals in the dose calibrator right before imaging, where the reduction in whole body activity shows the additional clearance of substance with respect to time post injection. This can only be avoided by comparing euthanized animals, where kinetics have been stopped or comparing quantification on a phantom level, i.e., on a designated mouse phantom. Another parameter that can cause a difference in resulting values is the amount of activity injected for imaging and for *ex vivo* biodistribution purposes, when different animals have been used for the two methods, even if the exact same time points have been analyzed. In imaging applications (especially tomographic ones) the required activity to achieve good statistics can be quite high (1 – 2 mCi), but in *ex vivo* biodistributions, it can be as low as only a few uCi. This can alter the binding profile of a specific targeting compound. This can be avoided by comparing imaging and *ex vivo* biodistribution results that come from the exact same animals (i.e., biodistribution is performed after the animals are imaged and euthanized). This factor is also less significant when planar scintigraphy is performed with γ-*eye*™ and β-*eye*™, where the required activity is much lower (only a few uCi). An additional advantage of low activity imaging is the metric scales that are more consistent between animals and humans (4).

As far as the parameters that affect each quantification method are concerned, several points must be considered. For quantification from imaging studies, the accuracy in relating the density of the detected photons and the concentration of the pharmaceutical in an organ, can be in principle, limited by instrumentation and processing factors. Photons that are emitted in the soft tissue can be absorbed or scattered before reaching the detector, the resolution of the imaging system can blur the activity distribution and partial-volume effects can occur or counting statistics can affect the temporal resolution of the system (19). However, the small size of the

animal models might present a challenge on the instrumentation part, but attenuation and scatter levels are quite low and thus are beneficial for quantification purposes (1). More specifically, the attenuation of detectable photons by soft tissue has been estimated to be up to 25% when imaging Tc-99m in rat-sized objects (so much less for mice) and scatter less than 10% for Tc-99m and rodent sized objects (20). In some cases, quantification through imaging can also be affected by the accurate definition of regions of interest, based on SPECT or scintigraphy. In such cases delineation through CT or planar x-ray might improve the quantification accuracy (21). However, in oncology applications, where tumors are grown subcutaneously and do not overlap with other organs, these deviations become significantly less important, and the quantification becomes more straight-forward.

These facts illustrate that, to provide trust-worthy quantification results in preclinical, oncology studies, many factors should be addressed with great attention. Imaging methodologies evolve in a very fast way and are becoming more accurate. Careful selection of the appropriate model system and best imaging modalities, as well as an optimal study design, are crucial points that determine the translational potential of a study (22). If parameters are kept optimal during all procedures, the analysis on specific oncology models show that the three presented methodologies present similar results ($p = 0.45 – 0.93$) and thus can be equally trusted. *Ex vivo* biodistributions present an accurate but time-consuming, animal-consuming quantification methodology.

While the value and accuracy of 3D imaging is well proven, the statistical analysis highlights the added value of 2D imaging, which has several practical advantages. It is known that the purchase and maintenance cost of 3D systems is high, and they are usually a core facility and cannot be used as a daily tool for testing multiple compounds. However, at the early stages of a new tracer development, small variations during synthesis procedure can have a significant effect on the biodistribution. Thus, fast methods that provide yes/no answers, using a minimum number of animals is desirable. Considering that the majority of published papers uses maximum intensity projection (MIP) images, which are almost identical to fast 2D imaging, we suggest this approach as a robust, fast quantification method, compliant with ethical guideline on animal use, respecting the 3Rs principle.

5 Conclusions

The comparison between the three methodologies, to evaluate organ accumulation and tumor targeting, shows a non-significant deviation between 2D and 3D imaging and a similar deviation between 2D or 3D and post-imaging *ex vivo* biodistributions. If the suggested parameters are given the necessary attention, all methods can be accordingly trusted. Non-invasive imaging methodologies present a much faster, more ethical, and more economical way to implement preclinical research, when compared to *ex vivo* biodistributions. Moreover, 2D imaging could potentially allow for acquisition across more research groups reducing, even more, the cost for some studies as the costly 3D imaging systems would not be required. Finally, the fact that quantification results in oncology models do not present significant differences between the tested methods, is a supporting factor for further improvement and a clear direction towards small-animal imaging, even using simple planar imaging.

6 Compliance with ethical standards

All applicable institutional and/or national guidelines for the care and the use of animals were followed.

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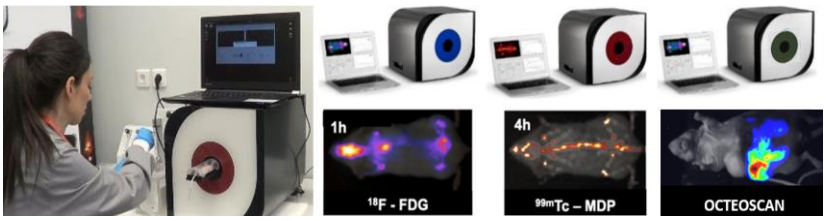
8 About BIOEMTECH

BIOEMTECH is allocated in the Technological Park of Demokritos Research Center, in Athens Greece. Having a strong academic background, the company provides unique, high-quality products and services in the field of drug research and biotechnology.

9. BIOEMTECH products: *eyes*

BIOEMTECH specializes in the design and construction of desktop, small animal imaging systems for pre-clinical, pharma, biotechnology, and medical research; Our trademark '*eye*' refers to compact and desktop devices, which transform lab desks into *in vivo* imaging labs, allowing easy and real time *in vivo* dynamic screening of radiolabeled compounds, providing unique information for imaging PET and SPECT isotopes, as well as fluorophores.



(Left) The γ-eye™ system in use, (Right) Top to bottom: β-eye™, γ-eye™, φ-eye™ systems, indicative images from the eyes with ^{18}F-FDG, ^{99m}Tc and Octeoscan, respectively.

10. BIOEMTECH CRO services

BIOEMTECH Laboratories offer a full preclinical platform, from *in vitro* studies to *in vivo* imaging. We provide small animal imaging services, for advanced experiments on multi-scale level for both diagnostic and therapeutic protocols, in our unique, fully equipped, and licensed laboratories that include:

- *In vitro* lab for cell studies
- Animal facility (mice & rats)
- Radiochemistry lab
- Imaging facility (micro-CT/SPECT/PET)

BIOEMTECH Laboratories: in vitro, radiochemistry, animal hosting and imaging labs, door-to-door.

All studies are in full accordance with the 3Rs principle (animal reduction, replacement, and refinement) and all international standards, while our personnel are specially trained on the undertaken activities (FELASA & radioactivity accreditation).

11. Contact Us

For more info do not hesitate to contact us.

BIOEMTECH

Lefkippos Attica Technology Park N.C.S.R. Demokritos, Athens, Greece,

+302130290586-7. info@bioemtech.com

sales@bioemtech.com

www.bioemtech.com