　
Beginner's Guide

A beginner's guide to imaging *in vivo* tumour biochemistry

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The Beginner's Guides are an ongoing series of feature articles in the magazine, each one covering a key technique and offering the scientifically literate but not necessarily expert audience a background briefing on the underlying science of a technique that is (or will be) widely used in molecular bioscience. The series will cover a mixture of techniques, including some that are well established amongst a subset of our readership but not necessarily familiar to those in different specialisms. This is a Beginner's Guide to imaging *in vivo* tumour biochemistry.

Using antimatter to look at cancer

Cancer can be defined as the uncontrolled proliferation of cells in the body that arise from genetic mutation. Cancer is a devastating disease, accounting for more than a quarter of all deaths in the UK (Cancer Research UK, https://www.cancerresearchuk.org/health-professional/cancer-statistics-for-the-uk, accessed May 2019). Over the last couple of decades, a detailed understanding of the molecular pathways associated with cancer growth, progression and metastases has led to the development of new and improved treatments that target these pathways. For example, the identification of the HER2 receptor, with the resulting drug, Herceptin, has produced long-term survival benefits in many women with HER2-positive breast cancer. We are able to characterize, visualize and measure these important biochemical biochemical processes in a living system using a powerful medical imaging technique called positron emission tomography (PET). Unlike other medical imaging technologies, such as computed tomography (CT) or magnetic resonance imaging, which provide detailed anatomical information, PET can reveal the functional processes that drive disease without the need for surgical intervention. In this article, we will discuss the basics of PET and the steps involved in developing 'smart' imaging probes to visualize *in vivo* tumour biochemistry using PET.

Cancer is a heterogenous disease, with multiple phenotypes observed both between different metastatic sites and within the primary tumour itself. Tumour heterogeneity has widespread implications for patient diagnosis, treatment selection and ultimately, how these tumours respond to therapy. Diagnosis is traditionally confirmed by biopsy following referral by a general practitioner, often after the detection of a suspicious

growth or the onset of symptoms. Biopsies are an invasive procedure in which a small region of the suspected lesion is surgically excised and examined under a microscope to assess the presence (or absence) of malignant cells. Tumour biopsies, however, hold a number of limitations: as only a small region of the tumour is sampled, a single biopsy may not capture the whole picture of the disease itself. In extreme cases, inadequate sampling can lead to the misdiagnosis and wrongful treatment of patients. In addition, acquiring tumour biopsies is an invasive procedure, and in some cases a biopsy cannot be obtained at all due to the location of the mass. By comparison, PET imaging can provide doctors with whole-body images of cancer biochemistry. This allows the assessment of both primary tumours and distant metastases. Seeing 'the whole picture' allows a more accurate diagnosis and improved patient care.

PET imaging works through the indirect detection of an antimatter particle called a positron, emitted from an unstable atom, known as a radionuclide. These radioactive atoms can be attached to a molecule of biological interest to produce a radiolabelled molecule known as a radiotracer. Following intravenous injection into a subject, the radiotracer accumulates at the selected biological target of interest and is detected by a PET scanner. A detailed description of this process is given in Figure 1.

Designing smart imaging probes

If properly designed, a radiotracer can allow us to visualize important molecular processes relating to cancer. Before designing the radiotracer, an informative biological target to image must be selected. This biological target is called a 'biomarker' and can be an indicator of normal

Figure 1. Preclinical PET imaging. Prior to acquiring a PET image, a radiotracer is injected into the subject (**1**). Radiotracers are made by attaching a radioactive atom (radionuclide) to a scaffold that targets a biological process of interest. As the unstable radioactive atom decays it emits a positron (the antimatter equivalent of an electron), which travels a short distance (~1 mm) before colliding with a near-by electron (**2**). Upon collision, these 2 particles undergo a process called 'annihilation', releasing 2 γ-rays at ~180 degrees to one another. The detection of these γ-rays by a PET scanner allows the origin of annihilation to be calculated, subsequently providing the precise location of the radiotracer. Computer processing enables multiple annihilation events to be processed, building a 3D 'map' of radioactivity inside the body—the PET image (**3**).
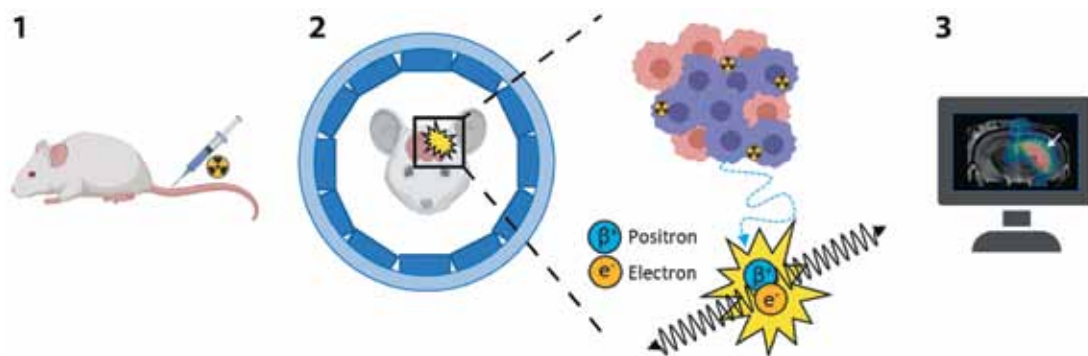
biological processes, disease processes or responses to an intervention, such as drug treatment. Important examples of biomarkers relating to PET imaging include receptors, enzymes, proteins and transporters. The data obtained by the measurement of a well-selected biomarker can then be used to answer important clinical or fundamental questions relating to cancer biology.

In a clinical setting, imaging will look to address a question regarding how to treat a patient. This could be 'does this patient have cancer?', 'how is the patient's cancer responding to their therapy?', 'will the patient's cancer respond to this type of therapy?' or even 'what is the cancer phenotype?' The different biomarkers exhibited by cancer cells can be targeted by PET probes to achieve selective uptake and accumulation in specific cancer types to answer these fundamental questions. This accurate characterization of the cancer allows treatments to be selected for individual patients rather than the population as a whole in a process known as precision medicine.

Successful visualization of the biomarker relies on 'contrast'. Firstly, this means that the target must be differentially expressed in cancer over that of the background tissue, with any background uptake of the radiotracer clearing within the lifetime of the radioactive atom. Without this clearance, contrast cannot be achieved and visualization of this biomarker will not be possible. Finally, for the visualization to be informative, the radiotracer must be delivered to, and specifically interact with, its biological target. This requires appropriate design and application of the radiotracer.

An excellent example of a PET radiotracer for cancer imaging is [^{18}F]FDG, a glucose analogue that is taken into cells by glucose transporters and phosphorylated by the enzyme hexokinase (Figure 2A). The replacement of a hydroxyl group by the radioactive fluorine isotope means that after intracellular phosphorylation the sugar becomes trapped, since the altered structure cannot be further metabolized. The increased glucose metabolism exhibited by cancer cells means that [^{18}F]FDG is preferentially taken up, allowing high contrast imaging of tumours. This is a perfect example of how a radiotracer can be used to measure altered cancer biochemistry, which may inform

treatment decisions. [^{18}F]FDG is now used in most modern hospitals for disease staging and confirmation of primary diagnoses. As we move towards more personalized medicine regimens, however, there is a requirement for probes not only to detect cancer but to distinguish cancers of a specific phenotype or aggressiveness. This requires the development of new, 'smart' imaging probes. In the design of these informative probes, a number of considerations must be made:

1. Where is the biological process occurring?

In order to image a biochemical process, the probe needs to be able to interact with the system under investigation. Delivering molecules and biological scaffolds to where you want them in the body is far from trivial. It is a problem that drug companies and their medicinal chemistry teams spend billions of pounds trying to solve. The same problems need to be addressed in PET imaging. Firstly, the radiotracer needs to get to the site of disease. This can be tricky as the complex *in vivo* environment means that many compounds are quickly cleared (removed from circulation) or broken down in the body. The compound may also need to cross physical obstacles such as the blood–brain barrier or a cell membrane. The correct selection and/or design of the radioactive probe is key to overcoming these issues. As a result, the design of the radiotracer will vary considerably depending on the location of your biomarker, not only within the body, but at the cellular level. The properties of a radiotracer designed for an extracellular target can vary dramatically from a radiotracer imaging an intracellular or transmembrane target, as discussed below.

2. What type of scaffold will I use?

Scaffolds that can be radiolabelled for the production of PET radiotracers include small molecules, peptides, antibodies and other proteins. Each of these scaffolds can target biomarkers to produce an informative image. Small molecules, such as inhibitors or substrates, can target intracellular proteins or enzymes. The intracellular location of the target means that the radiolabelled probe must cross the cell membrane. This may require
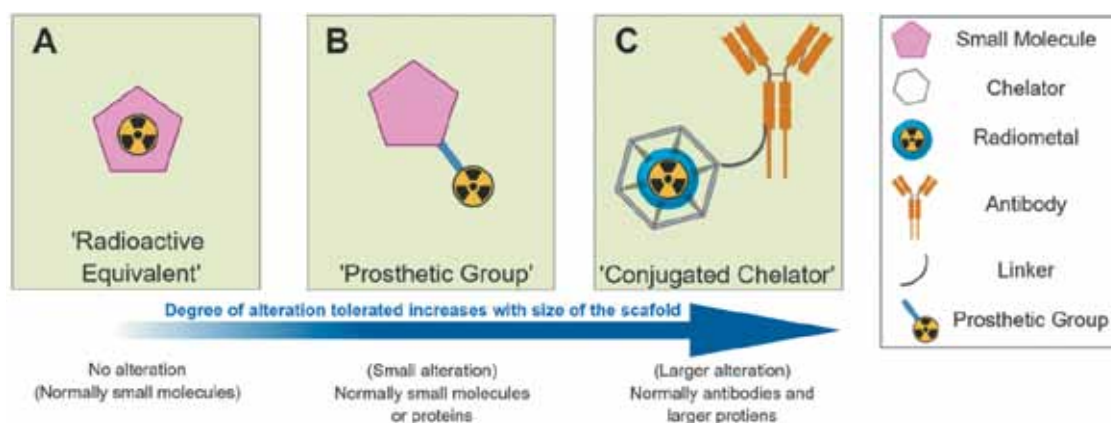
Figure 2. Different radio-labelled scaffolds and their applications. (**A**) [^{18}F]FDG is transported into cells where it accumulates due to metabolic trapping subsequent to phosphorylation by hexokinase. (**B**) A radiolabelled drug molecule crosses the cellular membrane and binds to its intracellular protein target. (**C**) Radiolabelled antibodies can bind to the extracellular domain of specific receptors.
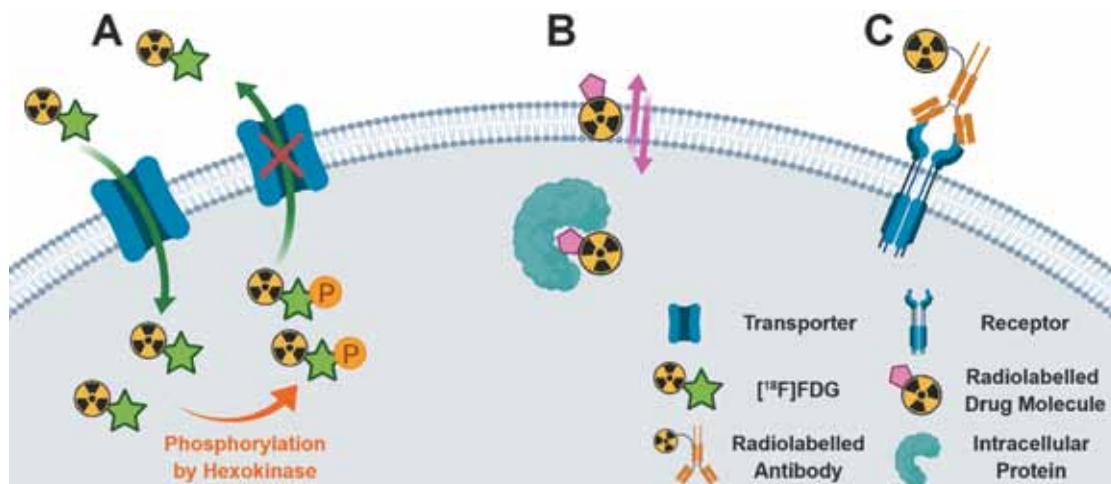
the design and tuning of specific properties of a small molecule, whilst radiolabelled endogenous species, such as sugars and amino acids, may have mechanisms for cell internalization, often via membrane transporters. The probe can accumulate in the cell by binding to its recognition site (Figure 2B). Alternatively, a substrate-based probe can undergo a modification that leads to its 'trapping' in the cell. This 'trapping' can be due to alterations leading to an inability to cross the cell membrane once it enters the cell (e.g., phosphorylation of FDG, shown in Figure 2A) or binding at another intracellular site. Small molecule cell transporter substrates can also be radiolabelled to detect differences in cell transporter expression.

Peptides, antibodies and other proteins are typically used to detect extracellular biomarkers. Advantageously, any issues regarding the cell membrane permeability of these scaffolds are negated for extracellular biomarkers. Radiolabelled versions of these scaffolds bind to specific receptors on the cell surface. This allows visualization of tumours exhibiting increased receptor expression compared with healthy cells (Figure 2C). This can inform a clinician if a patient is likely to respond to therapy targeting this particular cancer type, e.g., HER2-expressing tumours.

Synthesis of smart imaging probes

The synthesis of radioactive molecules (termed radiochemistry) has unique challenges, the most important of which is the synthesis and purification of the radiotracer within the time constraints of the decaying radionuclide. Production and purification should normally be completed within two half-lives of the radioactive atom (before 75% of the starting radioactivity has decayed) so there is enough radioactive material left to enable reliable imaging of the disease. Synthesis is further complicated when using large amounts of radioactivity. While the opening credits of The Simpsons may suggest that a biohazard suit and a pair of tongs are suitable protection for handling radioactive material, this certainly isn't the case for PET radionuclides! The γ-radiation produced is highly penetrating and permits the deep tissue visualization of PET probes. This means that standard organic chemistry procedures are impossible, or certainly not advisable! To overcome these problems, robotic systems have been developed to perform the desired manipulations in an automated fashion. These systems can then be placed in a heavily shielded compartment known as a 'hot cell' and controlled remotely.

The radiolabelling process depends on the scaffold

Figure 3. Different strategies for radiolabelling. As our understanding of how to deliver a radiotracer to its desired target progresses, radiotracer design can improve. In most cases, success will not be achieved at the first attempt. Novel radiotracers must be validated biologically (as discussed below). If *in vitro* or *in vivo* studies show that the compound is not fulfilling the requirements of an informative radiotracer, the design and synthesis processes must start again using the new information acquired.

chosen and the radioactive atom (radionuclide) selected. The half-life of your radionuclide should match that of the biological process you're interested in visualizing—you don't want all of your radiotracer to decay before the meaningful data is acquired. For example, the radionuclide fluorine-18 (half-life of about 2 hours) is very popular for labelling small drug molecules, as these compounds are rapidly removed from the blood and excreted. This allows contrast, and therefore visualization, of your target a short time after injection. Labelling an antibody with fluorine-18 would be impractical, however, as the radiotracer would completely decay before its removal from the blood, which can take many days. To visualize the target of the labelled antibody, better contrast is achieved much later. Therefore, a radionuclide with a longer half-life is generally used, such as zirconium-89 (half-life of about 3 days), so that contrast can be obtained. Once the radionuclide and the scaffold being labelled have been selected, a number of strategies are available to incorporate or attach the radioactive atom.

One approach is to produce a direct radioactive equivalent of your molecule of interest (Figure 3A). Advances in both radiochemistry and the robotic platforms used to perform the manipulations, have led to remarkable advances in our ability to incorporate radioactive atoms into structures of increasingly complexity. The radiolabelling can be performed using the radionuclide itself (e.g., fluorine-18 fluoride for fluorination) or by using radioactive reagents or building blocks (e.g., carbon-11 methyliodide for methylation) to synthesize the molecule. Alternatively, small molecules or peptides can have radioactive units known as 'prosthetic groups' attached to them to facilitate radiolabelling where direct substitution of a non-radioactive atom for one that is radioactive is not possible (Figure 3B). Larger species such as antibodies are amenable to more considerable alteration, whilst maintaining their original biological behaviour. This allows functionalization with larger structures such as chelators for coordinating radioactive metals including zirconium-89 or copper-64 (Figure 3C).

From radiosynthesis to imaging

Following the identification of an appropriate biomarker and development of the complementary imaging agent, cell-based validation must be completed to confirm the specificity of a probe to its target. Here, immortal cancer cell lines are incubated with the radioactive probe over a defined period of time. Scientists can then compare radiotracer accumulation or binding to cell lines with altered characteristics, for example, a drug-sensitive vs. a drug-resistant cell line, or cells expressing HER2 and those that do not. To check the selectivity of a probe for its target, blocking assays, competition assays or gene knock-down models are often used. Blocking and competition

assays are used to prevent target–probe interactions. If the probe is specific to the biochemical target that has been blocked, no radioactivity should be detected in the cell. Knock-down models work in a similar way. Rather than blocking the target, the target gene and therefore biomarker is removed completely. If the novel probe is specific for the gene that has been knocked-out, there should be negligible radiotracer binding – demonstrating selectivity of the probe to the specific biomarker of interest. These initial experiments are necessary before the probe can be translated into complex living systems. Redesign of the imaging probe is required if non-specific binding is observed in these relatively simple models.

If the new radiotracer is shown to specifically interact with the biomarker of interest in cell culture-based experiments, validation then progresses to small animal research and *in vivo* (live animal) imaging. Tracking of the probe in healthy rodent models is performed prior to disease models. Experiments in healthy mice are important to understand the healthy tissue clearance and subsequent excretion route of the novel radiotracer. The two main excretion routes are via the renal system (kidneys and urine) and hepatobiliary system (liver and the intestines). The excretion route of the probe will determine which cancer types can be imaged. For example, when using a probe labelled with fluorine-18, if radioactive signal is seen in the bone, this is a sign of possible defluorination, where the radioisotope has become detached from its scaffold.

A crucial part of radiotracer assessment *in vivo* is the selection of clinically relevant cancer models. The appropriate model could be a subcutaneously implanted tumour xenograft using tissue taken directly from a cancer patient; an orthotopic tumour model, where tumour cell lines are grown in the tissue from which they originate; or a spontaneously arising tumour generated in a genetically modified mouse, to name a few. Preclinical research provides vital information regarding the binding of a radiotracer to the target of interest, as well as information on how it is broken down. Dedicated small animal PET imaging scanners offer a platform for whole body, dynamic evaluation of these probes, often combined with CT for anatomical reference. If the probe has been successfully delivered to its target, a 'hotspot' of radioactivity will be seen in the reconstructed PET image (Figure 4), which also allows scientists to observe the stability of the radiotracer. For example, when using a probe labelled with fluorine-18, if radioactive signal is seen in the bone, this is a sign of defluorination, where the radioisotope has become detached from its scaffold.

It is important to understand the *in vivo* binding properties, toxicity and radioactive dose delivery of novel imaging agents for Medicines and Healthcare products Regulatory Agency (MHRA) approval, prior to testing in humans. These tests are imperative for the complete

Figure 4. Preclinical vs. human PET/CT imaging. **Left:** Preclinical PET/CT allows whole-body visualization of the radiotracer's biodistribution, dynamically in small rodent models. **Middle:** In current clinical PET/CT systems, whole-body imaging is not possible as the patient can not fit within the axial field of view of the scanner (usually 22 cm). This means only static scans, looking at the radiotracer distribution at a specific time, can be viewed. The clinical PET/CT image shown is a composite image from at least 2 or more bed positions. **Right:** Developments in technology (and a lot of money!) have led to the first total-body PET scanner.

Preclinical PET/CT   Clinical PET/CT   Total-body PET/CT

Soft pallet
Tumour
Kidney
Pancreas
Bladder
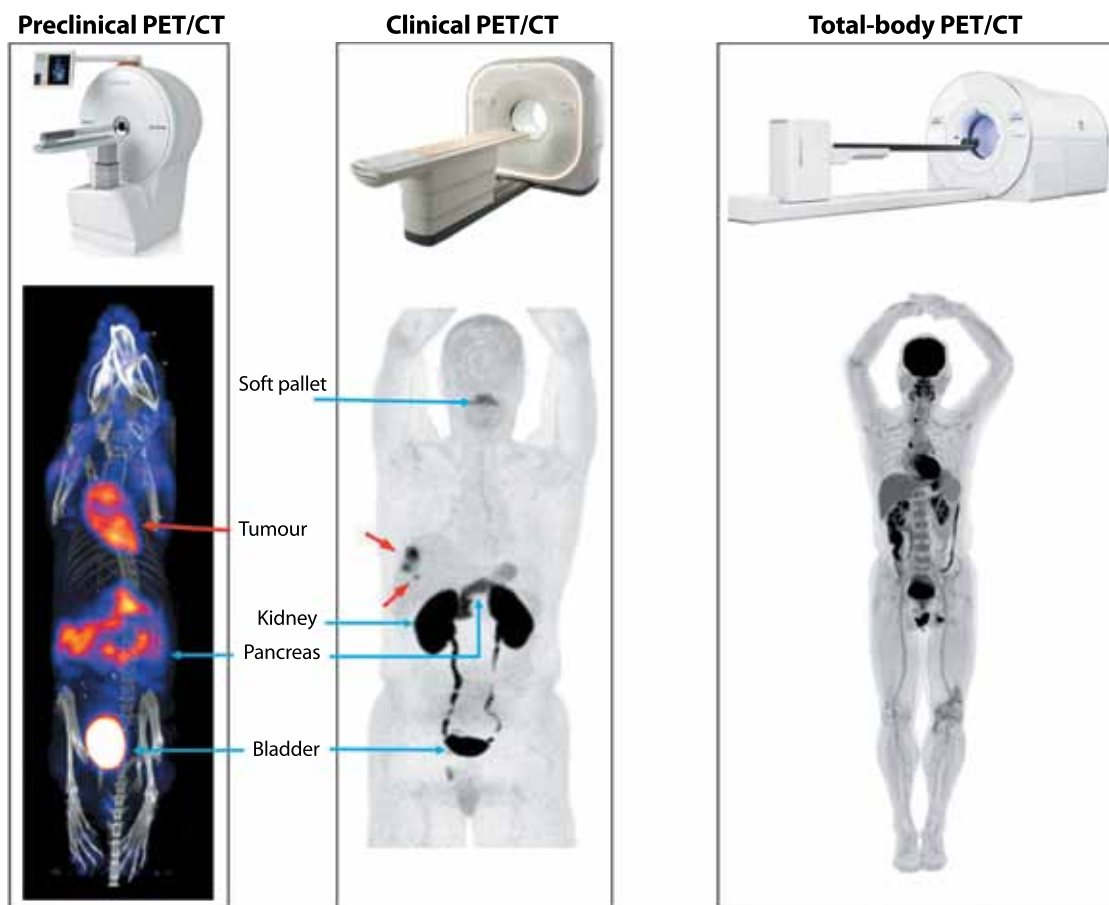
validation of novel imaging agents but it must be noted that a cell culture test or *in vivo* model will never replicate exactly what will be seen in humans. Clinical trials are crucial to evaluate the efficacy of novel imaging agents in the diagnosis and management of diseases.

The future of PET—antimatter matters

PET has advanced considerably from a technique used to simply visualize cancer lesions. One notable success is the emerging field of theranostics, which merges diagnostics and therapy into a single technique. As in conventional PET imaging, the first part of theranostic imaging is the diagnosis of a disease. A specially selected scaffold tagged with a PET radionuclide is injected into a patient where it will accumulate at the target of interest (if present). This accumulation of the radioactive probe will then be detected during a PET scan. If there is high accumulation of the probe, the next part of theranostic imaging is to deliver a targeted amount of therapy to the patient. For this, the same scaffold is used; however, the γ-emitting radionuclide is replaced with a corresponding isotope that emits α particles. These particles are highly charged and heavy atoms, and as a result they lose their energy in a very short distance, damaging the surrounding cells, for example, by causing

double strand DNA breaks. Specific delivery of these α particles to a cancerous lesion therefore gives a localized dose of therapy, which has been shown to be a highly effective treatment. This technique enables both selective imaging with the PET analogue, followed by therapy with the therapeutic equivalent. The most commonly used theranostic pair is technicium-99m and iodine-131 for the management of thyroid cancer. As well as thyroid cancer, theranostics has been extremely successful in the treatment of patients with neuroendocrine tumours and prostate cancer.

Excitingly, advances in scanner engineering have led to the development of the world's first total-body PET scanner (Figure 4). The total-body PET has an axial imaging field of view of 194 cm, almost 10-fold greater than a conventional PET/CT, allowing the entire body to be imaged at the same time. This milestone will provide previously unseen information on the whole-body dynamics of PET radiotracers. Lower doses, faster scanning and the ability to image at later time points will also be beneficial for patients and clinicians alike. The increased sensitivity that whole-body PET can offer will be particularly advantageous in cancer imaging, allowing the detection of small lesions that would previously go undetected.

Figure 5 (right). The development of a novel PET imaging probe. Following the selection of a suitable biomarker, radiolabelling of the appropriate scaffold is performed. The radiotracer is first validated in cells to examine specificity and selectivity to the biomarker, before being injected into a mouse model for preclinical *in vivo* PET imaging. If the outcome of these tests show promising results, testing of the radiotracer moves into human clinical trials, following MHRA approval.

Conclusions

Imaging tumour biochemistry *in vivo* is a complex process that requires a collaborative approach from a multidisciplinary research team. We have discussed the processes that are involved in selecting a biochemical target to image, synthesizing the probe and testing the radiotracer both in cells and *in vivo* prior to clinical translation (Figure 5). Non-invasive imaging of tumour biochemistry using new, smart imaging tools, places imaging scientists in a position to understand the mechanisms that drive cancer progression and the ability to treat the tumour whilst leaving healthy organs unaffected. The field of PET must strive to meet the demands of the medical community by delivering probes that can provide meaningful patient information, thereby improving the management and outcome of this devastating disease.

Target selection

Chemistry and Radiolabelling

In vitro validation

Small animal *in vivo* imaging models

Clinical translation

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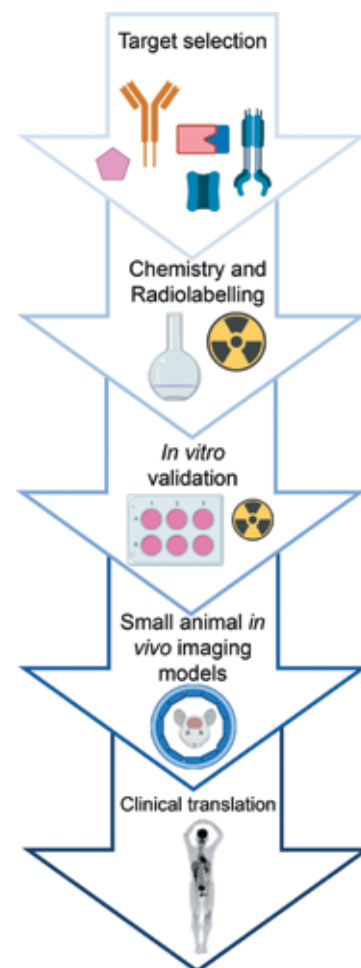
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