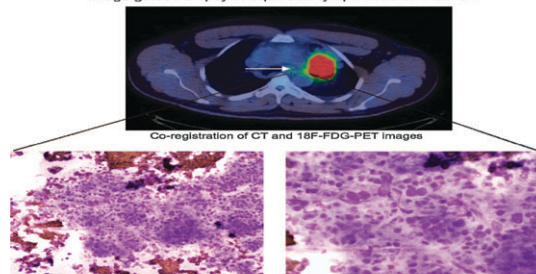


Image guided biopsy of equivocal lymph node on PET-CT



Haematoxylin and eosin staining of endobronchial ultrasound guided FNA of suspicious lymph node, 4L

**Q1** The challenges of integrating molecular imaging into the optimization of cancer therapy

G. S. Patel,\* T. Kiuchi, K. Lawler, E. Ofo, G. Fruhwirth, M. Kelleher, E. Shamil, R. Zhang, P. R. Selvin, G. Santis, J. Spicer, N. Woodman, C. E. Gillett, P. R. Barber, B. Vojnovic, G. Kéri, T. Schaeffter, V. Goh, M. J. O’Doherty, P. A. Ellis and T. Ng\*

We wish to highlight our attempt to link nanometre scale protein oligomerisation/interaction events to whole body imaging.

**Q2**

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REVIEW ARTICLE

**Q1 The challenges of integrating molecular imaging into the optimization of cancer therapy**

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 E. Shamil,<sup>a</sup> R. Zhang,<sup>g</sup> P. R. Selvin,<sup>g</sup> G. Santis,<sup>h</sup> J. Spicer,<sup>b</sup> N. Woodman,<sup>i</sup>  
 C. E. Gillett,<sup>ci</sup> P. R. Barber,<sup>j</sup> B. Vojnovic,<sup>jk</sup> G. Kéri,<sup>lm</sup> T. Schaeffter,<sup>n</sup> V. Goh,<sup>o</sup>  
 M. J. O'Doherty,<sup>p</sup> P. A. Ellis<sup>b</sup> and T. Ng<sup>\*ac</sup>

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We review novel, *in vivo* and tissue-based imaging technologies that monitor and optimise cancer therapeutics. Recent advances in cancer treatment centre around the development of targeted therapies and personalisation of treatment regimes to individual tumour characteristics. However, clinical outcomes have not improved as expected. Further development of the use of molecular imaging to predict or assess treatment response must address spatial heterogeneity of cancer within the body. A combination of different imaging modalities should be used to relate the effect of the drug to dosing regimen or effective drug concentration at the local site of action. Molecular imaging provides a functional and dynamic read-out of cancer therapeutics, from nanometre to whole body scale. At the whole body scale, an increase in the sensitivity and specificity of the imaging probe is required to localise (micro)metastatic† foci and/or residual disease that are currently below the limit of detection. The use of image-guided endoscopic biopsy can produce tumour cells or tissues for nanoscopic analysis in a relatively patient-compliant manner, thereby linking clinical imaging to a more precise assessment of molecular mechanisms. This multimodality imaging approach (in combination with genetics/genomic information) could be used to bridge the gap between our knowledge of mechanisms underlying the processes of metastasis and tumour dormancy and routine clinical practice. Treatment regimes could therefore be individually tailored both at diagnosis and throughout treatment, through monitoring of drug pharmacodynamics providing an early read-out of response or resistance.

† Micrometastases were originally defined as small occult metastases of less than 0.2 cm in diameter. Nowadays, the term refers to the spread of cancer cells in groups that are still so small they can only be seen under a microscope, and includes disseminated tumour cells that are present in peripheral blood, bone marrow or lymph nodes.

**1. Introduction**

Cancer therapies have evolved significantly in the past ten years with the advent of targeted treatments designed to a

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1 specific pathogenic process. Since the widespread adoption of  
the human epidermal growth factor (HER) inhibitor,  
trastuzumab (a monoclonal antibody to the extracellular domain  
of HER2) for HER2 overexpressing breast cancer, there has been  
5 a surge in the development of targeted, potential anti-cancer  
drugs. During drug development, only one in 10 000 compounds  
screened at the target localization stage will gain approval for  
clinical use. This process may take more than 10 years.<sup>1</sup>  
Furthermore, once the drug is within the clinical sphere, clinical  
10 outcomes rarely meet initial expectations.

As an example, single-agent phase II studies of epidermal  
growth factor receptor (EGFR/HER1) inhibitors have shown  
response rates only of the order of 5–15% in non-small  
cell lung cancer (NSCLC), head and neck squamous cell  
15 carcinoma (HNSCC), and colorectal cancer.<sup>2</sup> The tyrosine kinase  
inhibitors (TKI), erlotinib and gefitinib, are targeted to EGFR,  
and approved for use in non-small cell lung cancer.<sup>3</sup> However,  
response rates are less than 10% in unselected populations and  
overexpression of EGFR does not correlate with response to  
20 treatment.<sup>4</sup> Investigation of somatic gain-of-function mutations  
in EGFR, led to the discovery of a missense mutation L858R in  
the EGFR activation loop which facilitates gefitinib binding.<sup>5</sup>  
This and other activating mutations are correlated with a much  
improved response rate to TKI therapy, and have helped  
25 revolutionise the treatment regimens for NSCLC patients.  
However, further mutations conferring resistance (especially  
T790M) can occur which render EGFR resistant to first  
generation inhibitors.<sup>6</sup> Multiple mutations that confer resistance  
to the BCR-ABL tyrosine kinase inhibitor imatinib also exist in  
30 patients with chronic myelogenous leukaemia (CML).<sup>7</sup> In these  
CML patients, the genetic basis of additional molecular changes  
that occur and give rise to secondary drug resistance is frequently  
unknown. **Thus understanding the molecular genotype does not  
provide the complete explanation for resistance to molecule-targeted  
35 therapies.**

Here we propose that by combining different modalities of  
molecular imaging we can begin to delineate and quantify the  
specific molecular pathway alterations within the cancer  
patient at a subcellular level. The cancer genome or proteome  
40 is relatively plastic and can be reprogrammed, at different  
stages of tumour development, to carry out various cellular  
processes such as proliferation, invasion and metastasis, or  
reversion to dormancy.<sup>8</sup> This plasticity gives rise to spatial  
heterogeneity of cancer within the body and makes it  
45 challenging to fully assess treatment response. Molecular  
imaging provides a solution by mapping the spatial response

of the tumour to treatment within the individual and thereby,  
to monitor progress throughout the patient journey.

For instance, translational research may identify novel  
biomarkers in the malignant phenotype, which can be imaged  
5 by radioligands or tracers, designed specifically to target  
molecules intrinsic to oncogenesis. Examples of imaging  
biomarkers include tracers specific to hypoxia, angiogenesis,  
apoptosis and proliferation.<sup>9</sup> Although novel imaging bio-  
markers may provide a non-invasive functional read-out of  
10 the malignant genome or proteome throughout treatment,  
there are many challenges in the integration of these bio-  
markers into clinical practice.

## 2. Overview of challenges in the implementation of molecular imaging for improving therapeutic efficacy

Molecular imaging may provide an assessment of the temporal  
and spatial distribution of a probe or biomarker within a  
disease process. However the main challenge is to find the  
‘ideal’ imaging biomarker which should possess several  
20 characteristics, in order to accurately assess the effect of a  
therapeutic intervention and fulfil clinical utility. These are  
summarised in Box 1 below.<sup>10</sup> Several key areas are further  
discussed in the subsequent subsections (2.1–2.4). In particu-  
lar, in terms of clinical imaging, the second point regarding the  
25 activation state of a specific molecular target, which often may  
be concentration-independent, is seldom addressed in the  
literature. For this reason we have devoted a whole section  
(5) in this review to this topic.

### Box 1. Ideal features of a molecular imaging biomarker

- Ability to detect specific changes at the molecular (in terms  
of concentration) level
- Detection of the activated state of molecular target (which is  
35 independent of concentration), e.g. ligand bound or receptor  
dimerisation, as a read-out for monitoring drug efficacy
- Safe for human use
- High sensitivity and specificity to distinguish target from  
40 background or confounding signals e.g. slow tumour washout  
compared to normal tissue to maintain good signal-to-noise ratio

‡ Micrometastases were originally defined as small occult metastases  
of less than 0.2 cm in diameter. Nowadays, the term refers to the  
45 spread of cancer cells in groups that are still so small they can only be  
seen under a microscope, and includes disseminated tumour cells that  
are present in peripheral blood, bone marrow or lymph nodes.

### Insight, innovation, integration

We review novel, *in vivo* and tissue-based imaging  
technologies that monitor and optimize cancer therapeutics.  
Clinical outcomes have lagged behind development of  
targeted therapies. Combinations of imaging modalities  
55 should be used to assess tumour spatial heterogeneity, treatment  
response, and relate drug effects to dosing schedule.

Molecular imaging provides a functional and dynamic  
read-out of therapeutic response, from nanometre to whole

body scale. An increase in imaging probe sensitivity and  
specificity is required to localise (micro) metastatic‡ foci that  
are currently below systemic detection limits.

Image-guided biopsy is key in the multimodality imaging  
approach needed to bridge the gap between mechanisms under-  
lying pathological processes and clinical practice. Description  
of novel pharmacodynamic endpoints using this approach can  
provide early read-out of response or resistance.

1 (continued)

Box 1. Ideal features of a molecular imaging biomarker

- Detection of established ‘on target’ drug effects *in vivo* to assess drug efficacy and response
- Appropriate ‘off-rate’ or dissociation constant for adequate imaging but allows washout of biomarker prior to the next assessment.
- Rapid plasma clearance
- Low hepatic excretion to visualise liver metastases
- Rapid, simple chemical synthesis for tracer manufacture
- Inexpensive

2.1 Sensitivity and specificity issues

A difficult balance must be achieved whereby the tracer has a high affinity for the target, which may be present in very small

amounts, but low affinity for normal tissue to eliminate background noise. For example, somatostatin analogues have been in use for several decades to image gastroenteropancreatic tumours, as they have a high affinity for the somatostatin receptor (SSR)-2 which is overexpressed by these tumours and in the central nervous system.<sup>11</sup> Somatostatin is the ligand for SSRs but has a short half-life, which limits its use as an imaging agent. Somatostatin analogues are more stable, yet bind as the native ligand, thus conferring high specificity and sensitivity to this imaging modality. The PET analogues [<sup>68</sup>Ga-DOTA, Tyr3]octreotide or [<sup>68</sup>Ga-DOTA, Tyr3]octreotate are emerging as the new standard.<sup>12</sup> These analogues can be labelled with <sup>90</sup>Y or <sup>177</sup>Lu for use as radionuclide therapy with success as a palliative treatment, thus translating imaging to therapeutics in a single step.<sup>13,14</sup>

The capacity of a biomarker to identify specific cells within the tumour population may describe tumour characteristics,



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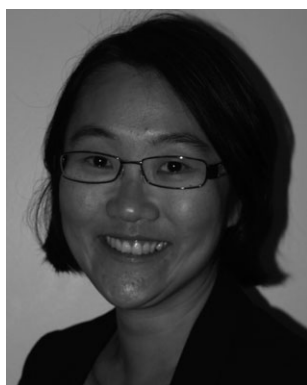
*recently received the McElwain Fellowship from Cancer Research UK for her research.*



T. Kiuchi

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*and biochemical techniques. Currently he is researching EGFR ubiquitination signalling downstream of homo and heterodimers of the ErbB family using FRET/FLIM assays and molecular biological experiments.*



V. Goh

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*programme focuses on the clinical imaging of the tumour micro-environment (vascularity, hypoxia, and water diffusion) and tumour heterogeneity with CT, MRI (including DCE-MRI, BOLD-MRI, and DW-MRI) and PET-CT with particular reference to gastrointestinal, renal and lung cancers.*



M. J. O'Doherty

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1 such as resistance to treatment. For instance, CD133+/  
CXCR4+ tumour-initiator cells have been shown to undergo  
a 2-fold increase as a subpopulation (from 3.5% to 7.5% of  
tumour cells) following *in vivo* cisplatin treatment of lung  
tumour xenografts in mice, as an indication of the intrinsic  
resistance of this cell population to chemotherapy.<sup>15</sup> While  
molecular imaging of CXCR4 in a murine model of breast  
cancer metastasis with [<sup>64</sup>Cu]AMD3100 has recently been  
published,<sup>16</sup> it is likely that its ultimate clinical use will  
be restricted to imaging cancer metastases in a only a few organs  
such as the lung, because there is a high uptake by normal stem  
cells at sites such as liver and bone marrow.

The relatively small numbers of ‘resistant’ or cancer stem  
cells within a tumour represents a further challenge to bio-  
marker sensitivity and whole body imaging. Potential cancer  
stem cells may constitute <1% of the tumour population.  
These cells may already be in the circulation, thus reducing the  
likelihood of identification by the biomarker. Even if the  
imaging probe has a sufficiently high specific affinity to bind  
to the target, significant signal amplification is likely to be  
required in order to detect minimal target concentrations  
(typically in the nano- to pico-molar range).<sup>17</sup> Imaging  
strategies and methods to amplify target signal are discussed  
in a subsequent section (see section 4.6 *Imaging drug resistance  
mechanisms including cancer stem cells*).

## 2.2 Spatial heterogeneity issues

Further challenges are faced in the attempt to image tumour  
biological responses at a microscopic scale, which will  
inevitably introduce another level of heterogeneity *i.e.* between  
individual tumour cells. Furthermore, these imaging techniques  
have a limited depth of penetration and do not inform on  
whole body distribution. There is scope to link whole body  
imaging (*e.g.* PET-CT) to imaging of pathological mechanisms  
within the tumour cells at a nanometre scale. The enabling  
technologies linking these two imaging scales will include  
image-guidance by the co-registration of different images

(*e.g.* PET and ultrasound (US)), to improve the current  
accuracy of sampling tumour-infiltrated lymph nodes, for  
instance. Image-guided biopsy may complement whole body  
imaging by improving the accuracy of assessment of response  
and recurrence, but is invasive. Tumour sites exhibiting poor  
response to therapy may be biopsied to define whether these  
cells exhibit a clonal change or a change in receptor expression.  
For instance, the difference or discordance in protein expression  
(*e.g.* HER2 status<sup>18</sup>) on cancer cells between the primary  
tumour and distant metastatic sites may correlate with a  
differential sensitivity to treatment (to be expanded on further  
under section 4.2 *Use of imaging to characterise tumour  
heterogeneity*).

Cancer patient management is guided by the classification  
of tumours into a variety of subtypes, representative of their  
pathology and stage, as described by light microscopy, bio-  
markers derived from antigen-specific immunohistochemistry,  
mutation and cytogenic analysis, and gene microarray data.<sup>19</sup>  
However, intratumour spatial heterogeneity may reduce the  
validity of this categorization. For example, nuclear  
polymorphism represents one of several characteristics used  
to determine grade in both invasive ductal carcinoma (IDC)  
and *in situ* breast cancer. Yet the commonly used grading  
systems do not recommend a minimum proportion of nuclei  
that need to be classed in the most marked pleomorphism  
group. How representative this is of the tumour as a whole is  
arguable. Conventional grading according to the modified  
Scarff–Bloom–Richardson method assigns a score depending  
on the highest level of nuclear atypia. Furthermore, detailed  
analysis and subclassification of entire DCIS lesions, by  
immunohistochemistry and microarray analysis, showed  
intratumoural biological diversity in 46% of all samples.<sup>20</sup> It  
is likely that histopathological analysis leads to an under-  
estimation of the total intratumour heterogeneity as only a  
small percentage of the tissue is examined. The use of  
endoscopic ultrahigh resolution optical coherence tomography/  
microscopy (resolutions of <4 μm axial and <2 μm



P. A. Ellis

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optical imaging and cell  
biophysics. Using FRET  
and FLIM techniques, his  
laboratory has established,  
in live and fixed tumour cell  
systems (including xenografts),  
imaging-based methods that

application of optical proteomics and novel clinical research  
strategies in the adjuvant and neoadjuvant setting.

can monitor post-translational modifications and protein inter-  
actions, both in space and time. By combining imaging with  
bioinformatics and network modelling, we are now adopting a  
multidisciplinary approach to understand the cancer metastatic  
process and its immunological control.

transverse)<sup>21</sup> is one of the imaging solutions that has been used to overcome this issue of inadequate tumour sampling.

Although molecular imaging may help delineate intra- and inter-tumour heterogeneity, these findings may create challenging clinical implications. For example, the smallest volume of the tumour expressing the imaged target which may warrant a change of treatment, and the implications of determining the molecular profile of the biopsied material from *e.g.* the secondary site, are issues which will have to be tackled in this context, as we move towards an era of multimodality and multi-scale cancer imaging.

### 2.3 Combining imaging of different modalities and (length) scales to follow treatment response

An early potential role for molecular imaging in cancer therapeutics is the measurement of tumour response to anti-cancer drugs. Current technologies are limited as the unit of response assessment is anatomical (CT/MRI/US) or a measure of metabolic activity which may be non-specific to drug activity (<sup>18</sup>F-FDG-PET). The imaging biomarker should be able to delineate established ‘on target’ drug effects *in vivo*, so that treatment efficacy and response may be assessed. The presence or concentration of a molecular target is not necessarily a read-out of target activity, which is more accurately depicted, in the case of the HER receptor protein-tyrosine kinases, by protein dimerisation, which in turn leads to phosphorylation and signal transduction *via* a variety of intracellular signalling cascades.<sup>22</sup> Except for one or two recent examples,<sup>23,24</sup> molecular parameters that delineate protein target activities, such as protein dimerisation, phosphorylation and other intracellular signalling events, cannot be obtained by whole body imaging and are best studied by specialised cellular and tissue imaging.<sup>25–29</sup> On the basis of our and other colleagues’ research findings in the HER field,<sup>26</sup> we maintain that for monitoring clinical response to therapies, molecular imaging would need to take into account the various processes of receptor activation, (*e.g.* ligand binding and dimerisation, which occurs at a nanometre lengthscale) in order to provide an accurate, functional read-out of drug efficacy. One of the key contributions we wish to highlight is our attempt to link these nanometre scale protein oligomerisation/interaction events (nanoscopy) to whole body imaging. Repeated imaging of various modalities may be necessary at different time points to obtain surrogate markers for treatment response, for instance in a neoadjuvant trial setting. However, this imaging approach may incur both financial costs and/or radiation dose concerns (for whole body imaging) as well as the requirement for repeat access to cancer tissues or cells from patients (for nanoscopic analysis) (discussed further in section 2.5 *Radiation and financial issues*).

A combination of imaging techniques, such as CT and PET or MR and PET may help delineate several different pathways sequentially or simultaneously. For example, clinical trials have demonstrated significant modification and improvements to external beam radiotherapy planning with the use of CT–PET imaging, as discussed below (4.1 *Combination strategies*). However, further work is required prior to routine incorporation

of this modality into treatment planning. The greater challenge is in the incorporation of radiotracers other than FDG into treatment planning. <sup>18</sup>F-FDG-PET scanning has established benefits in staging disease, as exemplified by numerous studies in cervical cancer, lung cancer, intracranial tumours and in assessing lower gastrointestinal recurrence.<sup>30</sup>

<sup>18</sup>F-FDG-PET has also been shown to be of benefit in the early assessment of response to therapy and as a prognostic marker for survival, *e.g.* for NSCLC, oesophageal cancer and lymphoma.<sup>31</sup> The initial assessment of tumour uptake using a semiquantitative uptake value (SUV) is of interest as a predictor for individual patient survival despite varying chemotherapy regimes.<sup>32</sup> Molecular imaging of tissue material from original biopsies may provide useful prognostic and predictive information on tumour biology which may relate to SUV.

Regardless of the issues surrounding use of combinatorial imaging modalities discussed thus far, a further ubiquitous challenge is present: the appropriate choice of biomarker for the diagnostic need. This will most likely vary between different tumour types and may even vary between different patients. Until recently *in vitro* basic biological research has established the mainstay of defining pathological biochemical and gene expression pathways. Molecular imaging holds the promise of evaluating physiological regulations of these pathways within their micro-environment. However, many different proteins are likely to be involved in tumour dynamics. It is not possible to image all those involved and therefore we must devise a strategy to elucidate key ‘nodes’ within these networks for evaluation in the patient. *In vitro* characterisation of protein–protein interactions has been integrated to build signal networks to model carcinogenic pathways or response to drug treatment, for example for EGFR.<sup>33</sup> ‘Nodes’ within these networks define key pathways which are integral for carcinogenesis or as a target for therapy. These networks may be used to generate prognostic molecular pathways which can be interrogated *in vivo* using molecular imaging, as discussed below (section 5.5 *Signalling networks to identify optimal drug combinations*).

### 2.4 A specific challenge in clinic-difficulties with quantification of images

The quantification of imaging signals requires careful consideration. The measured <sup>18</sup>F-FDG signal is the sum of 3 components: trapped intracellular <sup>18</sup>F-FDG, as well as the contribution from un-trapped <sup>18</sup>F-FDG in intracellular and intravascular spaces. In particular the last two components are strongly related to flow related effects.<sup>34</sup> The perfect biomarker for PET based research would be a radioactive tracer that is not rapidly metabolised and is trapped in the tumour or tumour environment, thus increasing its signal with time. Unfortunately the perfect marker does not exist. For instance, with a marker such as fluorothymidine, significant metabolism occurs such that debate exists as to whether correction for the metabolites is required to assess proliferation, the function that is being measured. This also raises the question as to whether visual assessment, semiquantitative assessment or true quantitative assessment is needed. Full quantitation increases the complexity

1 of the examination, often requiring the acquisition of an input  
function and scaling this input function to arterial blood  
radioactivity measurements. If the tracer is metabolised these  
measurements require correction for the amount of the  
5 metabolised product, thus altering the shape of the input  
function. Most PET centres do not have this ability. Therefore,  
in order to translate research into routine practice, the  
technique needs to be simplified, using visual or semiquantitative  
measures *e.g.* semiquantitative uptake values. Even for  
10 these simplified measurements standardised quality control  
(QC) and quality assurance (QA) is essential in order to enable  
different centres to assess the data using a common method.  
PET-CT has made major strides in establishment of common  
QA/QC for clinical trials with FDG within Europe.<sup>35</sup> These  
15 guidelines have provided a basic standard, but for more complex  
studies, the level of QA and QC requires escalation beyond these  
criteria.

Further issues regarding the quantification of imaging  
signals are faced within multinational biomarker studies.  
20 The Society of Nuclear Medicine in the United States of  
America (USA) has adopted similar guidelines to Europe.  
However, even within Europe, where there is a purported  
common European Clinical Trials Directive, the application of  
the directive is variable. This particularly applies to the  
25 investigational medicinal product dossiers required as part of  
the clinical trial authorisation for new radiotracers. The  
research and development process in the USA is different  
and leads to difficulties developing major biomarker studies  
for patients around the world.

30 These limitations for PET-CT data acquisition are being  
addressed such that pan European studies are carried out with  
data transfer to either a central facility for Europe or to “core”  
labs in individual countries, thus enabling multicentre research  
with FDG. Data quality is improved further with attention to  
35 detail in image processing methods, data acquisition, phantom  
data and daily, monthly and annual QC. The quantification of  
alternate tracers to FDG depends on the manufacturing sites  
available and the complexity of the image analysis to be  
performed.

40 Quantification from CT and MRI techniques is also a  
challenge. The signal of both DCE-CT and DCE-MRI is the  
sum of both intravascular and extravascular contributions,  
and dependent on flow and rate of vascular leakage. The signal  
of DW-MRI is affected by intravascular flow, the extra-  
45 vascular space volume, presence of macromolecules, and  
cell density.<sup>36</sup> Kinetic modelling approaches used for DCE  
techniques may allow quantification of this signal but make  
assumptions that may not necessarily hold in all cancer types  
or normal tissue. Measurement robustness remains an issue;  
50 this is affected by acquisition technique, particularly where  
signal to noise is reduced, though less so where a percentage  
change is being measured rather than absolute values. A further  
challenge is translating this to the whole body level. The  
coverage of DCE techniques depends on spatial resolution  
55 and temporal resolution, *e.g.* a typical coverage of 4 cm is  
achieved for a temporal resolution of less than 3 s for  
DCE-MRI for a single sequence.<sup>37</sup> Whole body DW-MRI  
is being assessed for staging and response assessment but  
quantification remains exploratory.

## 2.5 Radiation and financial issues

1 Imaging based on ionising radiation, such as X-ray, CT, and  
PET has a defined cancer risk and repeated imaging for  
pharmacodynamic end-points may lead to unacceptable levels  
5 of radiation exposure. However, this risk is still likely to be  
small when related to the overall lifetime risk of cancer in a  
normal population of 1 in 4 and the long term risks of  
chemotherapy and radiotherapy. Clinical radiation experts  
are cognisant of the issues related to radiation burden and in  
10 systems with financial constraints, keep a tight control over  
the amount of imaging performed. Furthermore, there is  
constant review, alongside manufacturers, of dose reduction  
strategies to achieve the same result. Patient acceptability  
and feasibility for repeated imaging must be paramount,  
15 and assessment of such must be included within the design  
of imaging clinical trials. The number and type of imaging  
interventions required must be rationalised in order to avoid  
these patient and financial costs. Cumulative radiation  
dose must be calculated for the entirety of the proposed  
20 treatment regime for modalities such as CT and PET which  
utilise ionising radiation. The radiation dose may be tempered in  
a number of ways.

A more targeted approach appropriate to the therapeutic  
effects should be considered. For instance, the efficacy of  
25 external beam radiotherapy is attenuated in areas of tissue  
hypoxia. Areas of low oxygenation undergo less necrosis on  
treatment as radiation-induced DNA damage is reliant upon  
oxygen. Hypoxia imaging, with <sup>18</sup>F-FMISO has already been  
shown to predict the response to radiotherapy in head and  
30 neck and NSCLC patients, and may play a role in the future in  
delineating disease for radiation boost, or reducing radio-  
therapy treatment in areas where it is likely to be ineffectual.<sup>38</sup>  
It is also possible that hypoxia imaging agents could be used  
to assess whether an improvement to tissue oxygenation has been  
35 achieved by an intervention, thus reducing the requirement for  
a dose boost.

The use of more specific imaging strategies for efficient  
response assessment should also be considered as alternatives  
40 to CT for anatomical response, in order to reduce total  
radiation dose. If ionising radiation imaging strategies are to  
be integrated into clinical practice, dose reduction strategies  
should be employed to maintain as low a radiation dose as  
possible. The use of lower injected activity and longer imaging  
45 times may reduce radiation dose from PET with the same end  
diagnostic result. The biologic effect of radiation dose is  
measured in millisieverts (mSv) and is calculated by multi-  
plication of radiation dose to organ, relative biological  
effectiveness and a tissue weighting factor. The radiation  
50 dose for most fluorine labelled tracers using 3–400 MBq is  
approximately 8 mSv for PET and 10 mSv for a body CT. The  
risk of inducing fatal cancer is 0.05/Sv or for a standard FDG  
PET 18 mSv scan, is approximately 1 in 1000 ( $18 \times 0.00005 =$   
 $0.0009$  or 1 in 1000). This figure has to be related to a 1 in 3  
55 natural lifetime risk for cancer. Cancer patients are at a higher  
lifetime risk for secondary malignancies due to anti-cancer  
therapy, *e.g.* radiation from external beam and internal  
delivery, and chemotherapy. The radiation risk should be  
weighed up against the potential benefit of imaging.



1 Justification of the use of radiation in molecular imaging for  
routine surveillance is equivocal, and is lacking for screening.<sup>39</sup>  
An appropriate evidence base must be established for the  
imaging intervention to detect treatable disease at an early  
5 stage and/or improve patient outcome, over and beyond the  
risk of secondary malignancy associated with the radiation.  
These databases have yet to be established for novel imaging  
strategies such as molecular imaging/imaging with novel PET  
probes.

10 Affordability and availability are two further challenges to  
the integration of molecular imaging to clinical practice.  
Nuclear imaging with PET is one of the most developed  
modalities for molecular imaging, and may be eminently  
translated to the clinic, due to the established use of  
15 PET imaging in lymphoma and NSCLC, for example.<sup>40,41</sup>  
However the routine use of PET imaging in a variety  
of tumour types for diagnosis or staging is not supported  
by evidence from randomised controlled trials (RCT),  
let alone the use of PET for screening. The current  
20 standard cost for a whole body <sup>18</sup>F-FDG PET scan is  
high; approximately £1000 in the UK. If molecular imaging  
utilising PET is to be integrated into clinical practice, this  
may entail multiple scans at an early stage in disease,  
thus increasing costs exponentially. Although a cost-benefit  
25 analysis is preferable, in reality, it is difficult for such  
interventions which may unpredictably change cancer  
therapy, and for which there are few RCTs to provide an  
evidence base.

The use of molecular imaging to select appropriate initial  
30 therapy and accurately assess disease response has the  
potential to reduce current costs significantly. For instance  
ineffectual drugs may be stopped early, saving not only the  
drug cost, but also producing health benefits in terms of drug  
toxicities, quality of life, in-patient admissions, and may even  
35 allow patients to return to work. For example, <sup>18</sup>F-FDG-PET  
scanning has been shown to improve selection of patients  
for hepatic surgery of colorectal liver metastases.<sup>93</sup> This  
study demonstrated a risk reduction in the number of futile  
laparotomies from 45% to 28% using PET-CT compared to  
40 CT, potentially saving costs of surgical interventions, inpatient  
stays and patient morbidity. Further large RCTs are required  
to calculate the cost benefit of carrying out PET scans,  
especially as many of the imaging modalities discussed thus  
far are research technologies. The ongoing Risk Adapted  
45 Therapy for Hodgkin's Lymphoma trial and a Cancer and  
Leukaemia Group B study are being carried out to address the  
benefit of PET imaging in reducing treatment intensity  
compared to standard high dose therapy or escalating therapy  
in those patients not responding to treatment, based on the  
50 FDG PET result. Data from studies such as these may  
help quantify the cost benefit of PET imaging. Within these  
studies, the acceptability and feasibility of multiple imaging  
for the patient must also be addressed. Molecular imaging  
with PET holds great promise in optimising cancer therapy,  
55 especially in the arena of surveillance and risk-adapted  
therapy.

In the following sections, we discuss examples of *in vivo* and  
*in vitro* molecular imaging modalities within the context of the  
challenges described above.

### 3. Various established and investigational imaging methods

Current imaging systems are based on the interaction of  
electromagnetic radiation or ultrasound waves with body  
5 tissues or fluids. High frequency electromagnetic radiation in  
the X-ray spectrum is ionising and may be tumourigenic in  
itself.<sup>94</sup> PET and nuclear medicine imaging systems have  
higher functional sensitivity compared to magnetic resonance  
imaging (MRI) which is more sensitive than X-ray systems  
10 such as CT.<sup>9</sup> Examples of established and novel imaging  
techniques are summarized in Table 1.

Until recently modalities such as CT and MRI have been  
used in diagnosis, staging and assessment of response  
to treatment by measuring the volume of disease. Gross  
15 macroscopic changes lag in time following alterations at the  
molecular level.<sup>95</sup> The Response Evaluation Criteria in Solid  
Tumours (RECIST) criteria, based on unidimensional tumour  
measurements, is the established method for assessing disease  
burden in clinical trials. However, structural changes may be  
20 non-specific, *e.g.* due to inflammation or malignancy. For  
example, the efficacy of cytostatic targeted therapies cannot  
be assessed on structural data alone.<sup>96</sup>

A combined approach, integrating the metabolic sensitivity  
of FDG PET with the anatomical spatial resolution of CT, is  
25 increasingly used in clinical practice. This has been validated  
for use in staging, detection of residual disease, and to assess  
response to treatment.<sup>97</sup> Using NSCLC as an example,  
PET-CT has improved staging accuracy, reduced futile  
thoracotomy rate and improved radiotherapy planning.<sup>98</sup>  
30 Recently, Positron Emission Tomography Response Criteria  
In Solid Tumours (PERCIST) have been introduced<sup>99</sup> which  
combines the quantification of anatomical changes (RECIST)  
with those developed by the EORTC PET response group.<sup>100</sup>

Although CT and MRI are mainly static techniques, emerging  
35 techniques, such as dynamic contrast enhanced (DCE) CT,<sup>101</sup>  
DCE-MRI<sup>102</sup> and DW-MRI have allowed quantification of  
vascularization and water diffusion respectively. Over the last  
decade, DCE-CT and DCE-MRI techniques have been explored  
40 in Phase I and II studies of anti-angiogenic and vascular  
disrupting agents to provide evidence of a mechanistic, anti-  
vascular effect.<sup>103</sup> Studies employing DW-MRI for response  
assessment are emerging.<sup>104</sup> Multiparametric approaches for  
example with MRI encompassing information on anatomy,  
45 perfusion, and cell density and proliferation has the potential  
to offer earlier, and more precise, information on treatment  
response in the neoadjuvant setting than RECIST.<sup>105</sup>

### 4. Current state-of-the-art cancer imaging applications

#### 4.1 Combination strategies

In clinical practice, the combination of various modalities such  
as PET and CT, have been shown to improve oncological  
55 imaging, especially for diagnosis, staging, response  
assessment, guiding biopsy and radiotherapy planning. In  
the USA, PET-CT is now included in NCCN practice  
guidelines in 21 cancers. In radiotherapy PET-CT has been

1 **Table 1** Current and promising research imaging modalities

Imaging modality	Contrast agent	Therapeutic intervention assessed	Benefits
<b>X-Ray based imaging</b>			
5 Computed tomography (CT)	Density of varying body tissues (variable absorption of X-Rays)	All therapies, and routinely used in clinically trials as end-point (RECIST criteria). Used for image-guided biopsy and radiotherapy planning	Routine practice, widely available, standardized. Good spatial resolution
10 Dynamic contrast enhanced (DCE)-CT <sup>42,43</sup>	Iodinated agents (time-to-peak enhancement correlates with tumour perfusion and vascular permeability)	Anti-angiogenic or antivascular agents, <i>e.g.</i> non small cell lung cancer (NSCLC)	Assesses on target drug effects, can be incorporated into readily available technology (CT)
10 CT colonography <sup>44</sup>	Tissue density (X-ray) and contrast agents	Colonic screening -detects polyps > 10 mm	Less invasive compared to colonoscopy, suitable for elderly patients, concomitant staging
15 Full field digital mammography <sup>45</sup>	Tissue density (X-ray)	Used as screening tool for breast cancer, in combination with computer assisted detection	Reduced radiation dose, improved sensitivity for dense breasts, tomosynthesis (3D visualization)
<b>PET</b>			
20 <sup>18</sup> F-FDG-PET ± CT	Uptake of <sup>18</sup> F-FDG, analogue of endogenous glucose	Response to imatinib in gastro-intestinal stromal tumours (GIST), prediction of response to chemotherapy in NSCLC, oesophageal and colorectal cancer. <sup>46-48</sup> Prognostic capacity in lung, oesophageal and thyroid cancer <sup>41,49</sup>	Clinically approved.  Quantification of tumour metabolic activity possible by SUV. <sup>50</sup> Predictive of treatment response with non-cytotoxic agents, <i>e.g.</i> imatinib. Improved biomarker for clinical response compared to RECIST
25 <sup>18</sup> F-FDG-positron emission mammography (PEM)	Uptake of <sup>18</sup> F-FDG	Identification of DCIS <i>vs.</i> invasive breast cancer	90% sensitivity for tumours less than 1 cm in size <sup>51</sup>
30 <sup>18</sup> F-FMISO-PET & <sup>18</sup> F-FAZA (Hypoxia imaging)	<sup>18</sup> F fluoro-misonidazole nucleoside ( <sup>18</sup> F-FMISO), and <sup>18</sup> F-fluoroazomycin arabinoside (faster clearance compared to <sup>18</sup> F-FMISO)	Predicts treatment response for radiotherapy in NSCLC and head & neck cancer, <sup>38</sup> clinical imaging of head and neck patients. <sup>52</sup>	Identifies hypoxic tumour tissue which is resistant to DNA damage by radiation or chemotherapy.
35 <sup>18</sup> F-FLT-PET (Proliferation imaging)	3'-deoxy-3'- <sup>18</sup> F-fluorothymidine (FLT) to infer rate of cellular proliferation	Response to chemotherapy in breast cancer and radiotherapy in pre-clinical models <sup>53</sup>	Non-invasive measurement of proliferation, especially relevant for non-cytotoxic drugs. (correlates with Ki-67)
<sup>18</sup> F-annexin V-PET (Apoptosis imaging)	<sup>18</sup> F-annexin V	Apoptosis imaging in animal models <sup>54</sup>	Lower uptake in the liver, spleen and kidneys compared to <sup>99m</sup> Tc-annexin V
40 <sup>18</sup> F-FES-PET	<sup>18</sup> F-fluoro-17βoestradiol	Response to tamoxifen in breast cancer <sup>55</sup>	May be able to delineate differential expression of oestrogen receptors in primary <i>vs.</i> metastatic deposits
45 Acetate PET imaging	<sup>11</sup> C-acetate	Well-differentiated hepatocellular cancer, brain carcinoma <sup>56</sup>	Labels relevant endogenous compounds to monitor intrinsic biological processes. Low renal excretion, may be useful in urological cancer.
<sup>124</sup> I-PET	<sup>124</sup> I-antibody fragments, <i>e.g.</i> anti-HER2 antibodies and <sup>124</sup> I-annexin V	Anti-HER2 labelled diabody used to image HER2 + ve xenograft <sup>57</sup>	Longer half-life (100.3 h) facilitates imaging, and matches biological half-life of antibody fragments used for labelling, and allows imaging at late time-points
50 <sup>89</sup> Zr-PET	<sup>89</sup> Zr-antibodies, <i>e.g.</i> <sup>89</sup> Zr-U36(anti-CD44 monoclonal antibody)	Stage and detect lymph node metastases in head and neck cancer patients <sup>58</sup>	Long half-life (78.4 h), as above. May be better than <sup>124</sup> I for internalising antibodies as <sup>89</sup> Zr remains in the cell
55 <sup>68</sup> Ga-PET	<sup>68</sup> Ga-peptides, cancer stem cells and antibodies, <i>e.g.</i> <sup>68</sup> Ga-Fab2-herceptin	RGD peptides (bind to α <sub>v</sub> β <sub>3</sub> integrins) image angiogenesis. <sup>59</sup>	Non-invasive monitoring of angiogenesis. Clinical application in patients with HER2 + ve tumours.
<sup>64</sup> Cu-PET	<sup>64</sup> Cu-vascular endothelial growth factor	<sup>68</sup> Ga-Fab2-herceptin used to monitor HER2 as a target for Hsp-90 inhibitors, in clinical phase I trials <sup>60</sup> Imaging of angiogenic vasculature <sup>61</sup>	Images VEGF, angiogenesis regulator, and monitoring of response to VEGF targeted drugs.

Imaging modality	Contrast agent	Therapeutic intervention assessed	Benefits
1 <b>Table 1 (continued)</b>			
5 Radiolabelled drugs	<sup>18</sup> F-desatinib, <sup>18</sup> F-paclitaxel, tamoxifen, fluorouracil and <sup>13</sup> N-cisplatin <sup>62</sup>	Imaging of prostate xenografts with <sup>18</sup> F-desatinib. <sup>63</sup> Pharmacokinetics of labelled chemotherapeutics: biodistribution, metabolism, response, dosimetry.	Ideally combines treatment and imaging. May be used for the study of drug pharmacokinetics
10 <b>MRI</b> MRI	Tissue relaxivity	Used for locoregional staging <i>e.g.</i> breast cancer, rectal cancer for staging and surgical planning. MRI is superior to US/ mammography for assessing response to treatment <sup>64</sup>	Excellent soft tissue resolution, Non-ionising radiation (recommended for patients at high-risk of radiation induced DNA mutations, <i>e.g.</i> BRCA1&2 <sup>65</sup> )
15 DCE-MRI	Ga chelates (kinetic modelling assesses $K_{trans}$ , $k_{ep}$ , $v_e$ , $v_p$ )	Predicts response to chemotherapy in breast cancer <sup>66</sup> and chemoradiotherapy in rectal tumours, <sup>67</sup> Assess effects of anti-angiogenic agents in early trials <sup>68</sup>	Dynamic studies possible utilizing a widely-available technology
20 Contrast enhanced (CE)-MRI	Targeted Ga chelates binding to cell surface markers of angiogenesis ( <i>e.g.</i> VEGF, $\alpha_v\beta_3$ ) <sup>69</sup>	Assess pre-clinical effects <sup>70</sup>	Non-invasive assessment of the angiogenesis
20 CE-MRI	Ultra small particle iron oxide (USPIO) accumulation in macrophages	Evaluation of lymphatic drainage for detection of micrometastasis, <i>e.g.</i> breast, bladder & prostate cancer <sup>71,72</sup>	100% sensitivity for LN mets in breast cancer (in combination with FDG-PET) <sup>71</sup>
25 CE-MRI	Targeted USPIOs, <i>e.g.</i> to annexin V, <sup>73</sup> HER2 receptor <sup>74</sup> or stem cell markers <sup>75</sup>	<i>In vitro</i> and pre-clinical <i>in vivo</i> demonstration of targeting agent only. <sup>76</sup>	Non-invasive assessment of key metabolic processes in oncogenesis, and potential to assess response to cytotoxic chemotherapy or targeted agents.
25 Diffusion weighted imaging (DWI)-MRI	Water diffusion <sup>77</sup>	Biomarker of response to chemo-radiotherapy, time to progression and overall survival in malignant glioma, <sup>78</sup> response to neoadjuvant chemotherapy in breast ca <sup>79</sup>	Whole body DWI-MRI may compete with FDG-PET for evaluation of soft tissue and bony disease.
30 Blood oxygen dependent MRI (BOLD MRI)	Blood oxygenation	Surgical planning in cranial tumours <sup>80</sup>	Non-invasive imaging of tumour hypoxia, especially useful intra-cranially <sup>81</sup>
30 <b>MR spectroscopy</b> Proton MRS	Proton (H) <sub>1</sub> , allows quantitation of tissue metabolites containing (H) <sub>1</sub> , <i>e.g.</i> choline, amino acids, nucleotides, lipids	Prognostication and assessment of residual disease in gliomas. <sup>82</sup> Pilot studies in staging breast and prostate cancer <sup>83</sup>	Improved specificity and resolution, especially when combined with MRI.
35 Fluorine Spectroscopy	Fluorine <sup>19</sup> F spectroscopy allows quantification of exogenous <sup>19</sup> F containing molecules <sup>84</sup>	Measurement of chemotherapy response <sup>85</sup>	Quantitative information of drug uptake
40 Spin hyperpolarisation	Hyperpolarised metabolites of labelled proteins. <i>e.g.</i> <sup>13</sup> C-pyruvate, acetate or urea	Pre-clinical <i>in vivo</i> measurement of chemotherapy induced cell death using <sup>13</sup> C-pyruvate <sup>86</sup>	May be useful for non-specific measurement of treatment response
40 <b>Optical imaging</b> Bio-luminescence	Overexpressed luminescent protein, <i>e.g.</i> luciferase	Relapse and metastases in prostate cancer xenograft models <sup>87</sup>	Very sensitive with high spatial resolution.
45 Optical coherence tomography (OCT)/ microscopy (OCM)	Varying reflection of low-coherence light from tissues	Differentiation of DCIS from invasive malignancy intra-operatively <sup>88</sup>	Image resolution of <1 micron, represents tissue microarchitecture comparable to histopathology
45 Fluorescence imaging	Molecular probes which may fluoresce in presence of target protein	Matrix-metalloproteinase activity in murine models <sup>89</sup>	Functional imaging of oncogenic process, can be translated to MRI
50 Förster resonance energy transfer (FRET) assays <sup>28,29</sup>	Interacting fluorescent probes	Effect of chemotherapy on caspase activity, <sup>90</sup> multiphoton endoscopy <sup>91</sup>	Highly specific method of functional imaging
50 <b>Ultrasound</b> Conventional ultrasound (US)	Echogenicity of tissues	Most commonly used in breast cancer detection, staging, and for image-guided biopsy	Inexpensive, widely available, non-ionising radiation
55 US + microbubble technology	Contrast microbubble agents	Detection of tumour angiogenesis in animal models <sup>92</sup>	Enhanced signal from tumour vasculature
55 <b>Nuclear medicine</b> Conventional radiolabelled ligands	<sup>131</sup> I, <sup>111</sup> In, <sup>99m</sup> Tc, <sup>67</sup> Ga	Neuroendocrine imaging with <i>e.g.</i> MIBG, or radiolabelled octreotide <sup>13</sup>	Ligand specificity to receptors overexpressed on tumour
55 $\beta$ -Particle emitter	<sup>90</sup> Y	Peptide receptor radionuclide therapy <sup>11</sup>	Direct translation of imaging ligand for therapeutic benefit.

1 integrated into radiotherapy planning for NSCLC with a  
modification in the definition of gross tumour volume  
(GTV) treated and improvement in inter-observer variability.<sup>106</sup>  
Similarly, PET-CT was found to improve GTV definition  
5 compared to CT alone for patients with pancreatic carcinoma,  
potentially reducing the risk of geographical misses.<sup>107</sup> In the  
latter study both scans were acquired separately and the data was  
co-registered.

10 Various co-registration software programs have been  
written in order to register pre-procedural scans with real-time  
scanning, as discussed for TRUS-MRI of the prostate  
(section 5.2). Briefly, matching landmarks on both studies  
are graded, either manually, automatically, or both, in order  
15 to match real-time needle positioning to anatomy from prior  
images. Similar software application to PET-CT guided  
biopsy of intra-abdominal lesions has been shown to be  
feasible.<sup>108</sup> This area is particularly difficult to characterise due  
to motion artefacts of peri-diaphragmatic structures, *e.g.* liver,  
20 due to respiratory effort. Non-rigid algorithms can accommodate  
this movement but require powerful computers and are labour  
intensive for routine procedures. Tatli *et al.* used rigid  
algorithms and achieved technical feasibility for biopsy of liver  
lesions.<sup>108</sup> Finally, in the case of PET-MR image fusion in soft  
25 tissue sarcoma which lacks conspicuous anatomical features and  
deviation from the rigid-body model, point-based PET-MR  
registration using external markers is practical, reliable and  
accurate to within approximately 5 mm towards the fiducial  
centroid.<sup>109</sup> Thus accurate targeting for biopsy is facilitated by  
the co-registration of multiple image modalities.

30 Fundamental improvements in the way we apply imaging in  
clinic can potentially be achieved by combining imaging  
modalities at different resolutions. For instance, MRI-guided  
clinical staging and presurgical planning may in the future be  
combined with intra-operative fluorescence-guided surgery,  
35 through the development and approval of nanoparticles  
that are dually labelled for *in vivo* fluorescence and MR  
imaging of proteases.<sup>110</sup> Other examples include nanoparticles  
that contain a radionuclide (*e.g.* <sup>18</sup>F) and a far red  
fluorochrome; with the latter being amenable to imaging  
40 with fluorescence-mediated tomography *in vivo*, and at micro-  
scopic (sub-micron) resolution *ex vivo*.<sup>111</sup> Promising  
technologies have demonstrated the feasibility of combining  
three different imaging modalities, PET, MRI and optical  
imaging.<sup>112</sup> This study demonstrates the advantages of  
45 combining these strategies, *e.g.* 50 times improvement in soft  
tissue sensitivity compared to conventional MRI, thus  
drastically reducing injected tracer volumes and rigorous  
probe validation.

#### 50 4.2 Use of imaging to characterise tumour heterogeneity

Disseminated tumour cells may exhibit a very different  
phenotype to that of the primary tumour. They may consist  
of stem cells, which are resistant to treatment or may express  
55 antigens which allow escape from immune surveillance in  
order to seed at a distant site and establish metastases. The  
assessment of tumour heterogeneity becomes imperative at  
metastasis, as differential protein expression across tumour  
deposits may have implications on the treatment regimes used.

One clinical example pertains to the overexpression of  
1 the HER2 receptor in a variety of tumour types, the most  
prominent of which is breast cancer. It is overexpressed in  
approximately 25% of patients and is associated with a poor  
5 prognosis.<sup>113</sup> A number of therapeutic interventions have been  
designed in order to block the HER2 receptor, including  
trastuzumab and small membrane-penetrating molecules that  
compete with ATP at the intracellular tyrosine kinase domain,  
*e.g.* lapatinib. The decision to employ HER2 targeted treatments  
10 depends upon overexpression of the receptor, as detected by  
immunohistochemistry (IHC), or gene amplification as deter-  
mined by fluorescence *in situ* hybridization (FISH), usually on  
primary tumour tissue alone. Comparison of the HER2 status of  
primary and metastatic lesions by IHC reveals significant dis-  
15 cordance: 127 out of 342 patients, 90 having a HER2 positive  
tumour but HER2 negative metastases, and 37 having a HER2  
negative primary tumour but HER2 positive metastases.<sup>114</sup> A  
similar series has observed heterogeneity for HER2 amplification  
within the primary tumour site.<sup>115</sup> This discordance between  
20 primary and metastatic tumour site could alter management of  
metastatic disease but is often not uncovered due to the difficulty  
in obtaining repeated, invasive biopsies on patients with meta-  
static disease. An imaging modality which could characterize all  
systemic lesions would greatly aid effective patient treatment.

25 Radiolabelled tracers to the HER2 receptor have been  
developed by labelling monoclonal antibodies, antibody and  
peptide fragments for PET, SPECT and MRI imaging.  
Although full sized antibodies have been used, they are slowly  
cleared from the bloodstream due to their size. Thus labelled  
30 fragments are being developed.<sup>116</sup> The most successful of these  
to date are HER2 Affibodies and a fragment of trastuzumab  
labelled with <sup>68</sup>Ga using DOTA (1,4,7,10-tetraazacyclododecane-  
1,4,7,10-tetraacetic acid) as the chelating group.<sup>117</sup> PET imaging  
is preferred to SPECT as it is about an order of magnitude more  
35 sensitive, detecting molecules in the pmol L<sup>-1</sup> range. Affibodies  
are small non-immunoglobulin-affinity proteins which are proven  
tracers for molecular imaging.<sup>118 111</sup> In- and <sup>68</sup>Ga- labelled HER2  
affibodies have been used in patients to visualize HER2 positive  
metastasis using PET and SPECT imaging, in 9 out of 11  
40 locations.<sup>119</sup> Although this result is preliminary, the tracers were  
well tolerated and comparable to <sup>18</sup>F-FDG-PET. One of the  
patients examined was on trastuzumab therapy, which did not  
interfere with radioligand binding.

45 These examples illustrate the feasibility of HER2 receptor  
imaging *in vivo*. However, integration of this information into  
patient management represents a further challenge. For  
instance, high uptake of <sup>111</sup>In and <sup>68</sup>Ga-affibodies in the  
kidneys and liver exclude these important metastatic sites  
50 from functional imaging. The spatial resolution of the images  
is sufficient to detect whether a HER2-positive metastasis is  
present or not, but not to delineate spatial heterogeneity  
within that sample. The presence of an established targeted  
therapy such as trastuzumab indicates that HER2 detection,  
either in a primary or metastatic site, warrants treatment  
55 with the targeted drug. However, the proportion of HER2  
receptors detected within the tumour may be difficult to  
standardise and quantify *in vivo*, due to the limits of resolution  
with PET imaging. Current histopathological recommendations  
define HER2 positivity as greater than 30% of cells exhibiting the

1 receptor on immunohistochemistry, and equivocal if greater than  
10%.<sup>120</sup> However, until these guidelines were published, con-  
troversies existed in this established field regarding  
standardised operating procedures, and proficiency testing within  
5 the laboratory. The direct translation of this definition to a  
3 dimensional *in vivo* sample is fraught with further difficulties.  
Even if potential HER2 receptor positivity is defined as 10% of  
the tumour volume on imaging, questions still remain. Does this  
volume refer to the total tumour volume within the patient or  
10 specifically to the site where the receptor is detected?  
Furthermore, the clinical significance of the volume of HER2  
detected is not known as cancer databases have quantified HER2  
positivity from IHC or fluorescence *in situ* hybridisation (FISH)  
from tumour biopsies thus far. Large scale observational clinical  
15 studies are required to assess the prognostic significance of  
varying levels of HER2 receptor detection within a tumour  
sample on imaging, prior to establishment of guidelines regarding  
treatment decisions.

By introducing our combined modality and multiscale  
20 imaging approach, biological heterogeneity that exists both  
within primary tumours and between primary and metastatic  
tumours becomes a significant challenge for rationalising  
targeted therapies. For example, questions such as the smallest  
volume (number of voxels) of the tumour expressing the  
25 imaged target that persists in a patient following targeted  
therapy, to warrant a change of treatment, have yet to be  
defined in this context. Having set out to describe the advent  
of novel imaging techniques, *e.g.* both radionuclide-based and  
nanoscopic imaging (section 5) of HER receptor, applicable to  
30 whole body and cells/excised tissues, we do not yet know the  
full extent of the heterogeneity issues that may be brought to  
light by these new techniques. It would be crucial in the future,  
however, to take into account the additional information  
obtained using these techniques and then validate their use  
35 in informing treatment response or possible patient  
stratification.

### 4.3 Drug pharmacokinetics and pharmacodynamics

40 Drug pharmacokinetics (PK) describes the effect of the body  
on a drug, namely, liberation, absorption, distribution,  
metabolism, and excretion. Pharmacodynamics (PD) describes  
the effect of the drug on the body, including therapeutic effects  
and unwanted toxicities. These properties describe two key  
45 factors in drug therapeutics; namely, how much of the drug is  
reaching its target, and whether it is fulfilling its purpose.  
Definition of the relationship between PK and PD is essential  
to the rational delivery and targeting of therapeutic agents,  
especially for those drugs with established molecular effects.  
50 Temporal delineation of drug pharmacodynamics can inform  
on drug response, appropriate drug dosing regimens and can  
provide an early assessment of resistance to therapy.

Traditional PK endpoints include invasive assessment of  
drug serum concentration by a variety of methods including  
55 liquid chromatography and mass spectrometry, and PD  
endpoints are assessed on repeated tumour samples or  
surrogate tissue.<sup>121</sup> However, repeated tumour sample biopsies  
can be challenging from the practical perspective. Biopsies are  
invasive, and fixed in time and space. Many sites are not easily

accessible *e.g.* intracranial tumours or mediastinal lymph  
1 nodes. Repeated invasive biopsies whilst patients are on  
therapy may confer patient morbidity and are not always  
acceptable or feasible.

PD endpoints are often defined by the maximum tolerated  
5 (MTD) dose, which is determined in phase I clinical trials by  
exposing sequential patient cohorts to increasing doses of the  
drug until the toxicities are intolerable. The MTD is described  
as one dose level below the dose at which intolerable toxicity  
10 occurs.<sup>122</sup> Optimal biological dose (OBD) is arguably a more  
rational phase I trial endpoint in the case of targeted  
therapies.<sup>123</sup> OBD is defined by PD assessment of effective  
target modulation, and may be attained at doses substantially  
below MTD.

The number of targeted novel agents available has increased  
15 exponentially over the last decade, but the tools to assess  
real-time function *in vivo* are awaiting more effective translation  
to the clinic. Molecular imaging could be used to assess OBD and  
aid decision-making in terms of appropriate dosing schedule and  
20 regimen, thus reducing the need for multiple biopsies. These non-  
invasive markers could illustrate real-time patient heterogeneity  
and differential drug sensitivity, both at the drug development  
phase, and in routine practice.

### 4.4 Pharmacokinetic and pharmacodynamic imaging biomarkers: potential applications and limitations

PET imaging, of either a directly labelled drug or an iso-  
tope-labelled ligand, has commonly been used in the assessment of  
25 drug PK and PD.<sup>124</sup> The quantitative nature of PET  
allows determination of drug concentration in tissue, as low as  
 $1 \times 10^{-12}$  mol L<sup>-1</sup>. The radionuclides commonly used for PET,  
*e.g.* carbon, nitrogen, or fluorine, may be incorporated into  
almost any drug for tracer synthesis, and the short physical  
30 half-life of these tracers results in favourable radiation dosimetry.  
Chemotherapeutics and targeted drugs have been radiolabelled  
in order to address their biodistribution and pharmacokinetics, *e.g.*  
<sup>111</sup>In-PEGylated liposomal vinorelbine, <sup>64</sup>Cu-DOTA-cetuximab  
or <sup>64</sup>Cu-DOTA-trastuzumab.<sup>125,126</sup> These studies are also known  
35 as ‘microdosing’, or phase 0 studies, whereby less than 1% of the  
therapeutic dose is administered, so that toxicities are unlikely yet  
drug half-life, rate of absorption and excretion can be measured  
on repeat scans.<sup>127</sup> However, the drugs do not achieve therapeutic  
efficacy, as shown by <sup>111</sup>In-PEGylated liposomal vinorelbine in a  
40 murine model of colon carcinoma, and as the doses are so low,  
extrapolation to PK of the therapeutic dose can be difficult. For  
example, first pass metabolism, gastrointestinal transporter  
mechanisms, and plasma protein binding can all be very different  
at such a low dose. The assessment of drug PK *in vivo* remains in  
45 the pre-clinical arena. Although clinical translation holds the  
potential to tailor dosing regimens according to individual patient  
metabolism, significant further research is required in the  
pre-clinical arena, for instance, to improve chemical specificity  
or methods of extrapolation from microdosing studies.

In terms of PD biomarkers, molecular imaging already  
55 plays a role in the clinical field. For example, <sup>18</sup>F-FDG-PET  
can be used to predict response to platinum based chemo-  
therapy in patients with NSCLC, as discussed previously.<sup>47</sup>  
This study used a non-specific radiolabelled tracer in order to

1 assess tumour burden. However, more specific radioligands  
are under development. <sup>64</sup>Cu-DOTA-trastuzumab and  
<sup>89</sup>Zr-trastuzumab have been used to demonstrate the  
effects of heat shock protein-90 (hsp-90) inhibition on HER2  
5 expression.<sup>126,128</sup> Hsp-90 is a chaperone for the receptor  
tyrosine kinase HER2. Inhibition of hsp-90 allows ubiquitination,  
degradation and down-regulation of this oncogenic protein in  
tumours overexpressing HER2.<sup>129</sup> This example represents the  
development of an imaging biomarker to visualise the 'on-target'  
10 effects of a drug, and real-time assessment of downstream *in vivo*  
effects. <sup>89</sup>Zr-trastuzumab has been approved for use in humans  
and has already been trialled in patients with metastatic breast  
cancer. Therefore, this probe could be translated into the clinical  
environment.<sup>130</sup> This study included patients who were currently  
15 receiving trastuzumab, and the authors did not find that  
concurrent treatment with the non-radiolabelled drug interfered  
with detection rates.

However, there are several limitations. This study on  
metastatic breast cancer patients clearly demonstrated high  
20 uptake of the radiolabelled drug in the liver, which precludes  
the imaging of hepatic metastases. As this is a prime site for  
metastases from many tumour types, alternative tracer  
development may be necessary. A general limitation of these  
tracers is the lack of *in vivo* chemical specificity. A radiolabelled  
25 tracer cannot always be distinguished from its radiolabelled  
metabolites, thus confounding functional biomarker read-out.  
Accumulation of the tracer in tumour may depend on intrinsic  
characteristics, such as vascularisation and necrosis, as well as  
tracer binding, complicating the result. Thus far, molecular  
30 imaging has been shown to improve clinical drug response  
assessment, albeit in a non-specific manner.<sup>48</sup> However, in  
order to develop imaging pharmacokinetic and pharma-  
codynamic end-points, extensive clinical evaluation is required  
in order to assess whether the biomarkers measure drug effects  
35 and whether this translates into a clinically meaningful  
benefit.<sup>131</sup>

#### 4.5 Mechanisms of drug resistance

40 Cancer cells may exhibit drug resistance due to a variety of  
mechanisms. Germline factors may contribute and include  
examples such as polymorphisms in *MDR1*, a gene encoding  
for efflux transporter *p*-glycoprotein, that limit the access of  
drugs to the site of action, and mutations in the tumour  
45 suppressor gene, *p53* that inhibit apoptosis.<sup>132</sup> Chemo-  
therapeutic agents such as anthracyclines and taxanes are  
hypothesized to elicit drug resistance *via* mechanisms such as  
increased drug efflux, decreased drug influx, target modification,  
drug detoxification or modifications to apoptosis signalling  
50 pathways, increased drug inactivation, increased repair of DNA  
damaged by chemotherapy and enhancement of alternative  
survival signalling pathways.<sup>133</sup> Alternatively, cancer stem cells  
may exhibit inherent, epigenetic mechanisms of drug resistance,  
as discussed in a later section.<sup>134</sup> Potential imaging biomarkers  
55 (both at whole body and subcellular levels) that detect and  
quantitatively monitor these resistance mechanisms may be  
invaluable in implementing the concept of personalised medicine.

Currently available systemic treatment for cancer rarely  
eradicates all disease as exemplified in the neo-adjuvant

setting. In a recent study the rate of pathological complete  
1 response after neo-adjuvant chemotherapy was quoted as 27%  
for basal-like, 36% for HER2 positive, and 7% for luminal  
subtypes of breast cancers.<sup>135</sup> Tailoring the treatment regimen  
5 employed according to the molecular mechanisms of drug  
resistance may improve patient outcome, for both cytotoxics  
and targeted therapeutics.

#### 4.6 Imaging drug resistance mechanisms including cancer stem cells

*P*-glycoprotein (Pgp), a transporter protein, is a member of the  
superfamily of adenosine triphosphate (ATP) binding cassette  
(ABC) transporters. Pgp maintains chemical homeostasis,  
especially at protective sites, *e.g.* brain, testes, and can pump  
15 cytotoxics out of the cell irrespective of concentration  
gradient. Therefore it has been of interest as a biomarker for  
both SPECT and PET imaging.<sup>136</sup> However, Pgp activity is  
difficult to image directly as ligands are actively extruded from  
the cell. Therefore, Pgp activity is inferred by measuring the  
20 absence of the radiolabeled substrate in a protected site,  
with or without a Pgp inhibitor. Several radiolabeled drugs,  
including chemotherapeutics such as <sup>11</sup>[C]-paclitaxel and  
<sup>11</sup>[C]-daunorubicin, have been used in animal models but the  
only drugs to progress to clinical evaluation are <sup>11</sup>[C]-verapamil  
25 and <sup>11</sup>[C]-loperamide. These tracers have been chosen due  
to intrinsic chemical properties, such as high-signal to noise  
ratio and low signal contamination by their radiolabeled  
metabolites.<sup>137</sup> However, none have yet been used in patients  
with drug resistant tumours. As studies have linked Pgp  
30 expression to drug resistance and lower overall survival rates,  
this imaging approach may be key to assessing its function  
in multidrug resistant cancer, and thus requires further  
development.

It is likely that drug resistance of a small number of rare  
35 cancer stem cells is naturally present before treatment with  
anticancer agents. The selective pressure of drug treatment  
encourages clonal expansion of these cells. Sharma *et al.*  
examined the effects of supramaximal EGFR tyrosine kinase  
inhibition in the PC9 non-small cell lung cancer cell line, which  
40 carries an EGFR activating mutation in exon 19.<sup>138</sup> Exposure  
to the EGFR inhibitor, erlotonib at 50 times the treatment  
dose (IC<sub>50</sub>) resulted in cell death for the majority of parental  
cells. The small surviving proportion (~0.3%) of non-  
dividing, quiescent cells acquired non-mutational (genetic)  
45 resistance to the drug treatment (named drug tolerant persisters,  
DTPs). The cancer stem cell phenotype was pivotal to survival of  
erlotonib treatment. All DTPs express the cancer stem cell  
markers CD133 and CD24, whereas the parental PC9 tumour  
cells exhibit heterogeneous stem cell marker distribution, which is  
50 associated with sensitivity to drug treatment. A synergistic effect of  
using erlotonib with HDAC inhibitors to eradicate the parental  
and the majority of resistant DTP cell lines was demonstrated  
in this study. *In vivo* assessment of the stem cell phenotype and  
55 inherent or acquired mutations conferring resistance to treatment,  
by subcellular imaging, could aid in the rational design of  
treatment strategy to overcome these mechanisms.

Real-time assessment of resistance is especially important in  
the stem cell population as the drug-tolerant state may well be

1 reversible. For example, colorectal cancer patients who are  
resistant to the chemotherapy drug irinotecan may become  
resensitised to the drug on cetuximab (a monoclonal antibody  
to EGFR) treatment<sup>139</sup> A similar phenomenon has also been  
5 shown in patients who exhibit primary or secondary resistance  
to the chemotherapeutic, oxaliplatin. Treatment with  
cetuximab sensitises these patients to oxaliplatin.<sup>140</sup> These  
observations suggest a constant state of flux in the prevalence  
of a variety of resistant 'stem cells'. Repeated biopsies to  
10 identify these cells may not be feasible or acceptable to  
patients. Therefore imaging of the mechanisms involved in  
the reversible drug-tolerant state, is likely to be key to successful  
eradication or control of tumour burden.

The development of a drug-tolerant state may be overcome  
15 by the use of metronomic dosing schedules. This involves the  
continuous administration of classic chemotherapy agents at  
relatively low, minimally toxic doses, with shorter or no drug  
free breaks.<sup>141,142</sup> Metronomic chemotherapy has been shown  
to inhibit tumour angiogenesis, but may also positively  
20 modulate the immune response against cancer cells and also  
induce tumour dormancy.<sup>141</sup> Metronomic chemotherapy can  
be used in combination with conventional chemo-radiotherapy  
and/or targeted therapy, with positive responses reported  
in hormone-resistant prostate cancer,<sup>143</sup> recurrent ovarian  
25 cancer,<sup>144</sup> and recurrent /metastatic breast cancer,<sup>145</sup> amongst  
others. Such a dosing schedule may be employed for those  
tumours in which a high proportion of resistant cells have been  
demonstrated by molecular imaging. However, metronomic  
therapy is not yet established practice and further work is  
30 required in this field, to, for example, define the number of cells  
making up the 'resistant population'. This may be hindered by  
the spatial resolution or sensitivity of the imaging modality as  
well as tumour heterogeneity, as described above.

Whole body assessment of resistant or stem cell distribution  
35 faces many challenges. These cells may be present in various  
tumour sites in very small numbers, or in the circulation.  
Whole body, metabolic imaging systems such as FDG-PET  
are limited in spatial resolution to 2–5 mm, with a high  
sensitivity ( $10^{-11}$ – $10^{-12}$  mol L<sup>-1</sup>, *i.e.* picomolar).<sup>17</sup> In order  
40 to use metabolic imaging to detect stem cells above back-  
ground noise, the imaging probe would have to bind to several  
million cells in close proximity. For example, within a 1 cm<sup>3</sup>  
tumour deposit, one may expect over 1 billion cells. If, for  
instance, stem cells occupy 1% of this volume, this would  
45 entail approximately 10 million cells which may be detected by  
PET, but only if these cells are within close proximity. If the  
probe detects innate changes in DNA or mRNA, the number  
of targets per cell would be reduced drastically to 1–2, or  
10–1000 per cell, respectively. It is entirely feasible that the  
50 realistic volume of cancer stem cells in the body may fall below  
the detection limit of whole body imaging.

This challenge may be circumvented in a number of ways.  
Amplification of the signal emitted from the reporter probe  
could allow detection of a much smaller number of cells,  
55 assuming the probe itself has a high affinity, sensitivity and  
specificity. The imaging probe itself may be amplified by  
avidin–biotin amplification or the attachment of large  
numbers of radioactive molecules to the probe, assuming the  
probe has a high specific activity.<sup>146</sup> Sensitivity of the probe

may be increased using fluorescence dequenching but this  
imaging modality has not yet been applied *in vivo* for this  
indication.<sup>147</sup>

Furthermore, with the advent of gene therapy, stem cells  
have been labelled, transplanted and monitored with  
5 non-invasive imaging, in animal models.<sup>148–150</sup> These methods  
utilise imaging techniques such as MRI with nanoparticles or  
<sup>18</sup>F-FLT PET imaging of non-specific cell processes. Stem cells  
are identified either at a known location, *e.g.* at sites of  
transplantation or sites where they are known to reside, such  
10 as the subventricular zone of the hippocampus for neural stem  
cells.<sup>149</sup> The relevant technology for monitoring cancer stem  
cells *in situ* would require non-invasive imaging of an  
established stem cell marker or gene transduction with reporter  
gene technology. Although the latter method has been applied,  
15 *in vivo*, *e.g.* for human mesenchymal stem cells in large animals<sup>150</sup>  
or adenoviral mediated transgene expression in patients with  
hepatic malignancy,<sup>151</sup> many challenges are associated with this  
type of technology. The main issue surrounds the logistical and  
ethical concerns regarding the transplantation of cancer cells into  
20 patients, which we do not have scope to fully address here. One  
example of clinical reporter gene use in a patient with grade IV  
glioblastoma, treated with cytotoxic CD8+ T cells genetically  
engineered to express the PET imaging reporter, illustrates an  
area for potential clinical application.<sup>152</sup> However, this technology  
25 is in its infancy and much work is required before general clinical  
use can be considered.

The identification of established cancer stem cell markers  
could provide a more readily acceptable assay for identification  
of stem cells *in vivo*. For example, CD133/prominin, a cancer stem  
cell surface marker, was identified in mouse xenograft models,  
using a fluorescent-labelled monoclonal antibody and quantitative  
30 fluorescence-based optical imaging.<sup>153</sup> CD 133 is a glycosylated  
transmembrane protein which loses specific epitopes upon  
differentiation, thus enabling its use as a cancer stem cell marker  
35 for brain tumours, pancreatic, colon, bronchial and prostate  
cancer amongst many others.<sup>154</sup> Optical imaging of CSCs within  
subcutaneous xenografts was possible, as confirmed by FACS  
and immunohistochemical analysis. However, direct translation  
of this technology for tumours which are not anatomically  
40 superficial would be difficult. Although there are several potential  
methods of identifying CSCs within tumour bulk, those utilising  
signal amplification of established biomarkers, are most likely to  
be applicable to clinical practice.

The identification of CSCs poses significant challenges  
45 to systemic imaging modalities, partly due to the limit of  
sensitivity of resolution. Following, we discuss alternative  
options for imaging at the micro- or nano-metre scale.

## 5. Protein oligomerisation/interaction imaging—preclinical and clinical applications

### 5.1 Linking whole body imaging modalities to micro-/ nanoscopic imaging of subcellular mechanisms *in vivo*

Attempts have been made to adapt whole body imaging  
modalities for imaging protein activity and function in  
tumours. For example, the morpholino-[<sup>124</sup>I]-IPQA probe  
was developed to bind irreversibly to the ATP binding site

1 of activated EGFR kinase, but not the inactive form, in order  
to demonstrate specifically the active form of this oncogenic  
receptor *in vivo*. PET imaging established an increased uptake  
in high EGFR expressing cell lines, in mouse xenografts,  
5 compared to low EGFR expressing lines.<sup>155</sup> In addition, by  
examining the kinetics of the radioactive decay during  
washout, this technique was shown to distinguish tumour cells  
expressing a constitutively activated variant of EGFR  
(EGFRvIII), from its wild type counterpart.

10 The interrogation of protein–protein interactions at the  
nanometre scale has been shown with PET imaging of a split  
reporter in xenograft models.<sup>24</sup> A genetically engineered  
PET-reporter construct, encoding the herpes simplex virus  
type 1 thymidine kinase (HSV-tk), is split with the N- and  
15 C-termini attached to hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ )  
and the Von Hippel–Lindau (VHL) tumour suppressor  
protein, respectively. Interaction between HIF and VHL, leads  
to reconstitution of HSV-tk, which can be quantified by  
microPET using radiolabelled probes such as <sup>18</sup>F-FHPG and  
20 <sup>18</sup>F-FHBG. However, as this strategy requires the injection of  
a genetically engineered construct, it is not yet appropriate for  
*in vivo* imaging use.

MRI is an alternative to PET imaging at the cellular level.  
Iron oxide nanoparticles with super paramagnetic properties  
25 may be used as contrast agents in MRI as they cause changes  
in the spin–spin relaxation times of neighbouring water  
molecules.<sup>156</sup> Surface modifications for conjugation of  
radiolabelled chemicals or therapeutic agents, are easily  
carried out on nanoparticles as they have a large surface area.  
30 Therefore these particles may be used as contrast agents, or to  
deliver targeted therapeutics to tumour sparing normal  
tissue.<sup>157</sup> For example, an IgG antibody that is specific for  
the truncated and constitutively active form of EGFR  
(EGFRvIII), which is only expressed in glioblastoma multi-  
35 forme, was conjugated to iron oxide nanoparticle and imaged  
in murine models, after convection enhanced delivery  
(CED).<sup>158</sup> The targeted delivery of the antibody was also  
therapeutic since a significant decrease in glioblastoma cell  
survival was observed, alongside reduction in EGFR phos-  
40 phorylation on immunohistochemical analysis of these cells.

A chemical biology approach can provide the “tool-kit” for  
combining different imaging modalities to examine tumours  
*in vivo*. For example, the matrix metalloproteinases (MMP)  
are involved in tumour invasion and metastasis. Fluorescent  
45 dendrimeric nanoparticles have been coated with activatable  
cell penetrating peptides (ACPPs) labelled with gadolinium, in  
order to bind to, and visualize MMPs by fluorescence imaging  
and MRI.<sup>89</sup> Active MMP-2 and MMP-9 on tumours were  
located in transgenic models, with fluorescence imaging and  
50 MRI. Fluorescent images detected post surgical residual  
tumour, and MRI was able to detect the spatial distribution  
of MMP within the tumour bulk. Often the tumours  
were surrounded by bright edges on MRI, tunnelling into  
normal tissue, thus visualizing the invasive and potentially  
55 metastatic process. Fluorescence imaging is limited in  
terms of depth of penetration, and clinically, is only validated  
for use with superficial disease *in vivo*. This technology  
is eminently applicable to the clinic for pre- or intra-operative  
resection, and assessing metastatic potential for primary

tumours. For instance, a clinical application could involve  
direct visualisation of tumour cell migration to sentinel  
lymph nodes.

## 5.2 Linking whole body imaging to micro-/nanoscopic imaging of subcellular mechanisms on excised cancer tissues/cells

Whole body imaging of tumour pathophysiological processes  
such as hypoxia, angiogenesis, apoptosis and proliferation  
visualises disease processes with millimetre resolution. In order  
10 to link these processes to molecular mechanisms which are  
mostly based on subcellular protein modifications (*e.g.* HER),  
such as dimerisation, phosphorylation and downstream  
signal events must be visualised. One approach is to  
utilise whole body imaging modalities such as CT, PET,  
15 MRI or US to delineate disease at a whole body level and  
guide biopsy to specific sites of interest, where subcellular  
processes may be assessed by, *e.g.* FRET or optical imaging.  
This strategy represents complementary information that  
completes the description of the cancer molecular phenotype,  
20 when used with the *in vivo* methods described above, and  
the associated development of multiple tracers as imaging  
biomarkers.

Image-guided percutaneous biopsy is a well-established  
method in cancer diagnosis. For example, over the last 20  
25 years, routine methods for the diagnosis and staging of breast  
cancer have relied on percutaneous biopsy under ultrasound  
or stereotactic mammographic guidance.<sup>159</sup> Breast cancer  
screening leads to a much higher detection rate of breast  
anomalies or microcalcifications. Most of these anomalies  
30 are not palpable and require image guidance to obtain  
diagnostic material. Image-guided biopsy has increased the  
accuracy of non-operative diagnosis and differentiation  
between malignant and benign disease from 63% to 95%. In  
the meantime patient morbidity has decreased due to a reduction  
35 in the rates of open surgical biopsy. Ultrasound-guided biopsy  
is also valuable in the neo-adjuvant setting in order to stage  
axillary lymph nodes for malignant infiltration. Fine-needle  
aspiration or core biopsies are taken and the tissue assessed  
for nuclear or histological grade, hormone receptor and HER2  
40 status, in order to plan surgery, chemotherapy and targeted  
treatment. Newer methods of imaging in this setting include  
proton MRS of biopsy material, which has prognostic  
significance.<sup>160</sup> The chemical composition of cells may be  
measured from biopsy specimens with this method. However,  
45 2D spectroscopy is also being applied *in vivo*, as an adjunct  
to MRI in order to delineate pathology in the whole breast  
at a subcellular level.<sup>161</sup> This technology is in its infancy  
but could help to highlight areas of interest for biopsy and  
to plan surgical intervention.

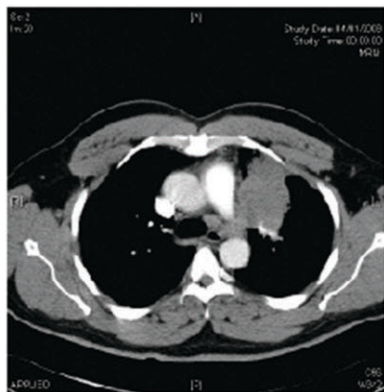
Another example of established image-guided biopsy  
techniques is the use of transrectal ultrasound (TRUS) as  
the gold standard method of visualising the prostate gland  
and determining potential malignant sites for biopsy.<sup>162</sup>  
55 However, up to 30% of cancers are missed at initial biopsy.  
A multiparametric MRI approach,<sup>163</sup> and co-registration of  
MRI and TRUS is being used to improve diagnosis and  
treatment, *e.g.* placement of brachytherapy beads. MRI has  
been used to guide transrectal and transperineal prostate



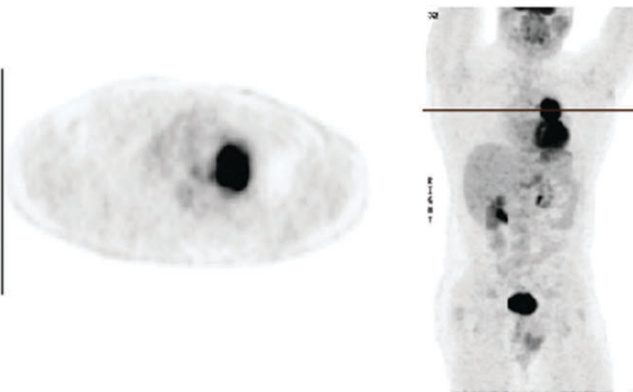
1 biopsy, but procedural times are prolonged due to the added  
complication of a magnetic field, and thus procedural costs are  
high.<sup>164</sup> Co-registration of preprocedural MRI images and

real-time TRUS solves these problems, and is potentially the  
most accurate method of image-guided biopsy for prostate  
cancer.<sup>165</sup> The accuracy of TRUS–MRI fusion system is

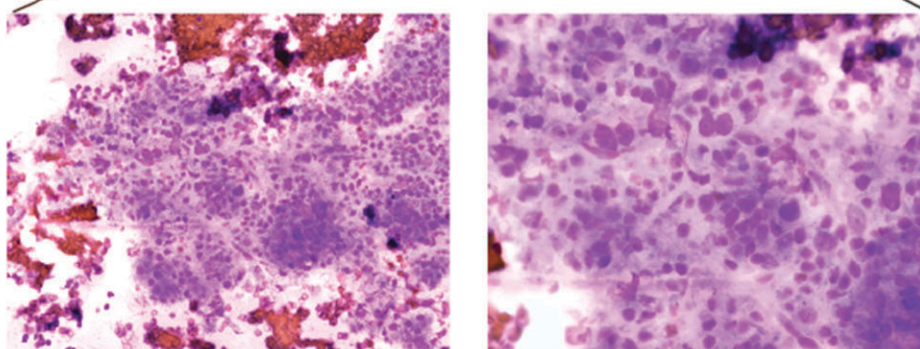
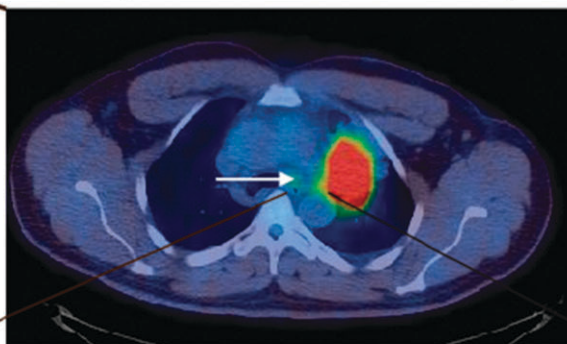
**A** CT imaging of lung tumour



18F-FDG-PET imaging of primary tumour, axial and sagittal planes

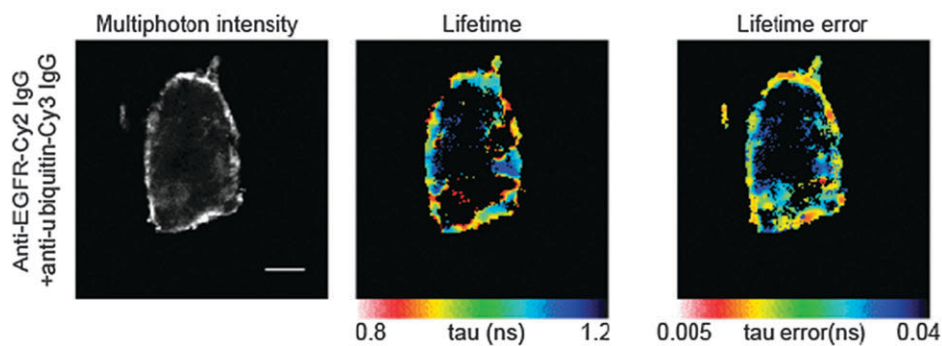


Co-registration of CT and 18F-FDG-PET images



Haematoxylin and eosin staining of endobronchial ultrasound guided FNA of suspicious lymph node, 4L

**B**



1 estimated to be 2.4 mm ( $\pm 1.2$  mm), which limits the detection  
size to lesions of  $5 \times 5$  mm.<sup>166</sup>

These methods have been applied for initial cancer diagnosis  
and to plan surgery or radiotherapy. However, immunohisto-  
chemical information from the tissue obtained by image-  
guided biopsy, may also be used to guide treatment decisions  
for chemotherapy and targeted therapy. For example,  
percutaneous <sup>18</sup>F-FDG-PET-CT guided bone biopsies have  
been shown to change the diagnostic staging, and thus alter  
planned treatment in over half of the cancer patients  
studied.<sup>167</sup> This group used repeated <sup>18</sup>F-FDG-PET-CT scans  
to position a needle in order to biopsy metabolically active  
bone lesions which were deemed equivocal on routine staging  
with CT, MRI or <sup>18</sup>F-FDG-PET.

Intended treatment was altered in 56% of patients ( $n = 20$ ),  
with a variety of tumour types. For example, patients were  
treated with palliative rather than curative intent, or with  
systemic therapy rather than surgery. Included in this category  
were patients for whom the image-guided biopsies were  
investigated for hormone receptor or HER2 status by immuno-  
histochemistry, which helped to decide on the appropriate  
treatment with anti-estrogen drugs and/or trastuzumab. This  
study illustrates the benefits of the combination of two imaging  
modalities in order to correctly biopsy equivocal sites, which may  
impact on treatment decisions.

Using a similar principle, our group is developing a protocol  
in order to combine information from pre-procedural  
<sup>18</sup>F-FDG-PET scans with real time endobronchial ultrasound  
guided transbronchial fine needle aspiration (EBUS-TBNA)  
of mediastinal and hilar lymph nodes in non-small cell lung  
cancer (Fig. 1). Smears and cell block preparations of  
EBUS-TBNA aspirates can be screened for EGFR mutations  
that can render the tumour drug resistant, such as the T790M  
mutation which can confer gefitinib resistance; as well as for  
quantifying protein-protein interaction of interest, *e.g.* EGFR  
ubiquitination or EGFR heterodimerisation with HER2  
(which has been shown to prevent EGFR dephosphorylation  
and signal termination by endomembrane-bound protein  
tyrosine phosphatases (PTPs)<sup>169</sup>) by fluorescence lifetime imaging  
(FLIM)/Förster resonance energy transfer (FRET) assays (see  
the following section). Subcellular imaging may be used to  
examine potential differences in these signalling events, at sites  
which are positive and negative on imaging with <sup>18</sup>F-FDG-PET.  
The success of treatment strategies may then be monitored (both  
spatially and temporally) by the changes in specific tumour cell

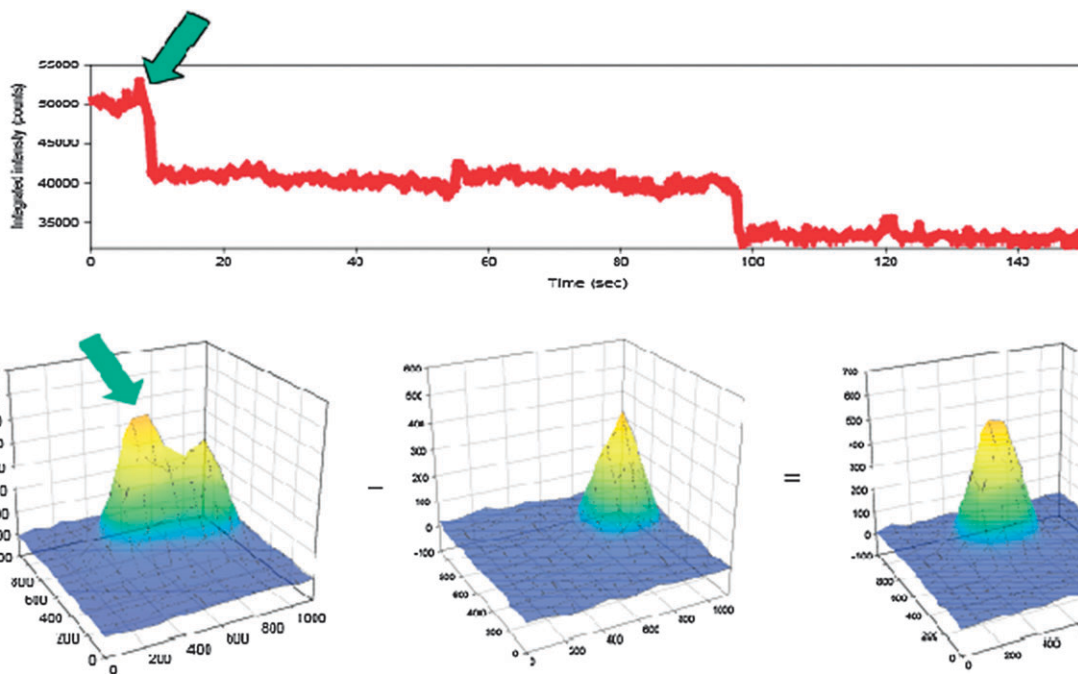
mechanisms. In the future, molecular imaging may be carried out  
on image-guided biopsy material, in order to apply the right  
treatment regimen to the right patient at the right time. The main  
challenge to this strategy would be the difficulty in choosing the  
site for biopsy and the need to minimise multiple biopsies, and  
hence the need to improve the accuracy of currently available  
image-guidance techniques.

### 5.3 Subcellular imaging of pathological mechanisms—preclinical applications

Optical imaging, fluorescence and bioluminescence studies  
best describe subcellular protein-protein interactions and/or  
protein modifications such as phosphorylation, at a high  
spatial resolution. For instance, Förster resonance energy  
transfer (FRET) imaging by fluorescence lifetime imaging  
(FLIM) has been used to quantify protein-protein interactions  
at the nanometre scale, *in vitro* and in live cells/  
animals.<sup>27–29,170,171</sup> This technique measures energy transfer  
from an excited donor fluorophore to an acceptor molecule in  
close proximity. In order for FRET to occur, molecules must  
be within nanometre proximity.<sup>172</sup> Therefore this technique  
may be used to visualise protein-protein interactions or protein  
modifications within cells. FRET-FLIM technology can be used  
to interrogate the malignant proteome, and its response to  
treatment, thus providing a functional insight which was  
previously the mainstay of analytical biochemistry. An example  
of application of FRET technology is the development of the  
so-called ‘Picchu’ (phosphorylation indicator of the CrkII  
chimeric unit) FRET probe which can be used to monitor EGFR  
activity in preclinical models.<sup>170</sup> Preclinical experiments using this  
FRET sensor demonstrated that the EGFR receptor remains  
active after endocytosis, until translocation to the perinuclear  
region. This effect may be modulated by its ligand, epidermal  
growth factor (EGF) and by TKIs.

Another example of using FRET imaging to monitor, in the  
preclinical setting, the pharmacodynamic response to therapy  
*in situ* is the application of the probe SCAT3 to monitor  
caspase-3 activity during tumour cell apoptosis subsequent to  
cisplatin or photodynamic (PDT) treatment.<sup>90</sup> This technique  
was repeated at varying time-points in order to observe drug  
effects on the tumour. Such an assessment of ‘on-target’ drug  
effects could greatly improve response assessment. However,  
currently FRET quantification is limited to superficial  
tumours. Development of instruments combining endoscopic  
cellular resolution imaging with technology to quantify

**Fig. 1** Endobronchial ultrasound guided transbronchial fine needle aspiration (EBUS-TBNA) to biopsy mediastinal lymph nodes in NSCLC. (A) Co-registration of images from CT and <sup>18</sup>F-FDG-PET combines spatial resolution of CT with the functional capacity of PET in order to stage this patient with non-small cell lung cancer. The middle image shows FDG uptake in the left upper lobe primary tumour (red spot) and very low FDG uptake in an adjacent left paratracheal lymph node, as demonstrated by the white arrow. The low FDG uptake in left paratracheal lymph node was not deemed to be significant but EBUS-guided transbronchial needle aspiration (TBNA) showed evidence of metastatic infiltration by non-small cell lung cancer (NSCLC). The H&E images (haematoxylin and eosin) show a mixed population of lymphocytes, but with a few groups of atypical cells. Within these cells, a high nuclear to cytoplasmic ratio, angulated and prominent nucleoli suggest malignant transformation. (B) FRET/FLIM assays are performed on samples obtained by EBUS-TBNA. EGFR ubiquitination is assessed by measuring FRET between anti-EGFR-Cy2 IgG and anti-ubiquitin-Cy3 IgG. Interaction between Cy2 and Cy3 results in a shortening of the lifetime of Cy2, as seen in the pseudocolour lifetime image. FRET efficiency was calculated using the following equation in each pixel and averaged per each cell. FRET efficiency =  $1 - \tau_{da}/\tau_{control}$ , where  $\tau_{da}$  is the lifetime of cells stained with both anti-EGFR-Cy2 IgG and anti-ubiquitin-Cy3 IgG and  $\tau_{control}$  is the mean anti-EGFR-Cy2 lifetime measured in the absence of acceptor. The lifetime error image on the far right illustrates the small error margins associated with this approach. Analysis was done using Bayesian fitting methods.<sup>168</sup> White scale bar represents 5 microns.



**Fig. 2** Photobleaching as a method of colocalisation with SHRIMP (single-molecule high resolution imaging with photobleaching) based upon fluorescence imaging with one nanometre accuracy (FIONA) SHRIMP techniques measure the distance between two dyes which are closer than the diffraction-limit. In the case shown, the dyes can just be resolved; by conventional microscopy, they are 330 nm apart, fit by two Gaussians. In order to determine this distance by SHRIMP, the sample is illuminated with light. Initially, fluorophores, for example, fluorophore  $F_1$  and fluorophore  $F_2$ , are bright, in total emitting with 2 units of intensity as shown on the graph in the lower panel (far left). Over time one of the fluorophores (e.g.  $F_2$ ) photobleaches, the intensity decreases to approximately 1 unit, and one of the Gaussian “hills” disappears, as shown in the middle graph. The position of the fluorophore which is still emitting,  $F_1$ , can be determined by fitting the centroid of the “hill”. The position of  $F_1$  can then be calculated by subtracting the image of both emitting— $F_1 + F_2$ —minus the emission after the photobleaching— $F_2$ , and fitting the centroid, as shown in the graph on the far right. The difference in the two centroids, i.e. 326 nm, is the resolution. This has shown to be effective down to 10 nm between two dyes.<sup>179</sup>

fluorescent lifetime and FRET, is underway and will increase potential clinical applications.<sup>173</sup>

Although FRET probes may be able to quantify direct protein–protein interactions, within 5–10 nm proximity, the spatial organisation of signalling proteins may be over the 10–250 nm scale on endosomal structures (including early endosomes to sorting endosomes and multivesicular bodies (MVBs)<sup>174</sup>). At this length scale, it may be difficult to quantify, using FRET imaging, the inter-receptor distance within homo-oligomers of EGFR,<sup>175</sup> and its heterodimer with other signalling receptors such as the c-Met receptor tyrosine kinase.<sup>176,177</sup> The spatial organisation of these receptors may provide significant insight into various mechanisms of resistance, for example anti-EGFR treatment by the acquisition of MET gene amplification.<sup>178</sup> In order to address this issue, a novel technique, is being applied, to map the 3D position of quantum-dot(QD)-labelled receptors in fixed breast cancer cells, within 3 nm accuracy, as shown in Fig. 2.

Modern super-resolution techniques can resolve the distance between identical molecules to about 8–10 nm; a significant improvement upon the conventional limit of resolution in visible microscopy, 200–250 nm. Two of the techniques are termed SHRIMP (single-molecule high resolution imaging with photobleaching) or SHREC (single-molecule high-resolution colocalization). These techniques are both based on

FIONA (fluorescence imaging with one nanometre accuracy), which is able to localise a single fluorophore within 1 nm accuracy. Despite the small size of the fluorophore (a few nanometres in width), its position is limited by diffraction which is approximately 250 nm. This is termed an airy function, and can be approximated by a Gaussian function, as shown in the lower panel of Fig. 2. Recent advances in detectors allow detection of the signal from an individual molecule so that the centroid of the Gaussian function can be located to 1 nm accuracy.

SHRIMP and SHREC utilize FIONA to detect the difference between two nearby Gaussian functions. In the case of SHREC, one chooses two dyes whose emission spectra are well separated from each other. Using an appropriate filter set, one can individually detect the location of each dye to FIONA-type accuracy. The difference in the centroids is the resolution.<sup>180</sup> In SHRIMP, the two dyes are exactly the same, and one relies on one of the identical dyes being turned off, generally by random photobleaching. The dye which is still emitting is located by FIONA to about one nanometre. The location of the original dye is obtained by subtraction of the emission immediately after photobleaching, from the image just before photobleaching. The resolution is then the difference of the position of the two centroids (Fig. 2). This procedure has been shown to work for molecules separated by 10–20 nm.<sup>179</sup>

1 Delineation of the spatial organisation of receptors at the  
subcellular level may help describe the tumour molecular pheno-  
type for selection of appropriate therapeutic agents.

#### 5.4 Subcellular imaging of pathological mechanisms—clinical applications

We have established FRET–FLIM assays in cell line models of cancer, fresh human tissues and formalin-fixed paraffin-embedded tissue (FFPE), as well as dynamic deep tissue imaging of cancer cells in murine models.<sup>28</sup> Fig. 3 demonstrates the translation of an *in vitro* protein–protein assay, measuring EGFR ubiquitination, in cell lines, to a dual antibody-based assay for quantification of EGFR ubiquitination in tissue, using FRET/FLIM technology. These images demonstrate *in vitro* assessment of functional EGFR modifications, *i.e.* ubiquitination, which is associated with downregulation and degradation of this receptor.<sup>129,181</sup> Cell lines with varying susceptibility to EGFR degradation (panel B), mimic varying tumour phenotypes. Thus, translation of these assays for use in patient tumour tissue may delineate a group of patients who are more likely to respond to drugs manipulating this pathway.

Besides whole tumour sections or image-guided biopsy material (Fig. 1), clinically these FRET–FLIM assays could also be applied to single metastatic cells, or the disseminating/circulating tumour cells (DTC/CTC), which confer a poor prognostic outcome in epithelial carcinomas, such as breast, lung and prostate.<sup>182</sup> The current validated methods for detection of DTCs rely on blood or bone sampling prior to immunocytochemical or molecular analysis.<sup>183</sup> Bone marrow involvement delineates the metastatic group more accurately in breast cancer patients, but bone marrow biopsy is invasive and the cells obtained are often not viable.<sup>184</sup> Circulating cancer tumour cells in the blood have been identified by a variety of immunological approaches, including identification of epithelium-specific antigens, *e.g.* cytoskeleton-associated cytokeratins, surface adhesion molecules, or growth factor receptors, and by molecular PCR-based techniques. The presence or absence of DTCs in the blood both before and after treatment has been shown to correlate with treatment response.<sup>185,186</sup> Furthermore, the presence or absence of radiological signs of disease progression can be combined with CTC counts in blood to improve the prediction of overall survival in metastatic breast cancer patients undergoing therapy.<sup>186</sup>

The presence of micrometastases in the form of DTCs may confer a poor prognosis as the cells may exhibit the same characteristics as cancer stem cells. They are resistant to therapy disseminate and grow at alternative sites and express many of the same surface markers as cancer stem cells, *e.g.* CD44, cytokeratin 19, and EpCAM.<sup>187,188</sup>

Molecular signatures obtained from the analysis of CTCs have also highlighted certain markers which may predict tumour dormancy.<sup>188</sup> Tumour dormancy is a phenomenon by which tumour cells evade eradication to become active many years later. The immune system has been demonstrated to play an important role in both animal models and in patients. For example, T-cell activation is strongly correlated with overall survival in patients with colon cancer, independent of primary tumour size or nodal status.<sup>189</sup> Further

research is being carried out in order to characterise the malignant phenotype of these cells. This information may then lead to clues as to how to target these resistant cells, and thus eradicate minimal residual disease. For example, DTCs in the bone marrow have been shown to overexpress urokinase-type plasminogen activator receptor and the extracellular matrix metalloproteinase inducer.<sup>190</sup> These targets are amenable to both imaging and for therapeutic potential. Imaging modalities which can monitor these cells *in vivo* would be of value to accurately gauge risk of relapse, and requirement for adjuvant treatment.

#### 5.5 Signalling networks to identify optimal drug combinations

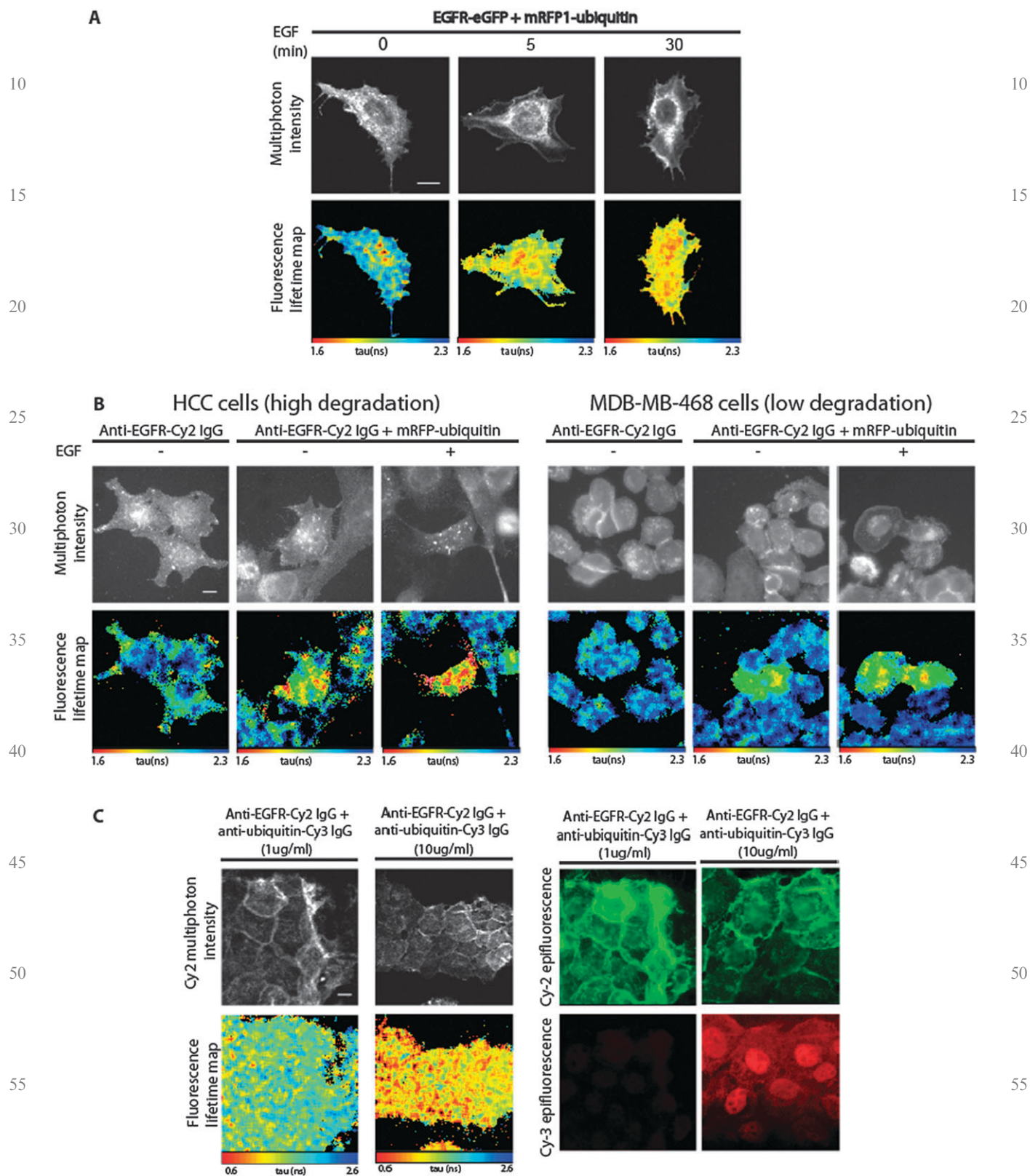
The response to treatment and the number of therapies required to eradicate a tumour has been mathematically modelled and integrated into signalling networks. These networks take into account biological tumour characteristics, such as the level of cell turnover, the rate of mutations, increased tumour size, and biological fitness for clonal expansion, as described mainly, by *in vitro* research.<sup>191</sup> Information derived from *in vivo* molecular imaging techniques could feed into these networks and greatly improve their capacity for prediction of treatment response and to design optimal drug combinations.

For example, a breast cancer patient who had kinome sequencing that revealed approximately 100 000 somatic point mutations would need 4–5 non-cross-resistant drugs to battle her cancer, according to this model.<sup>192</sup> Computational modelling has been used to create receptor tyrosine kinase co-activation networks to model the complex and dynamic interactions involved in chemoresistance.<sup>193</sup> Receptor co-activation describes the simultaneous activation of two or more receptor tyrosine kinases in order to maintain robust intracellular signalling in the face of perturbations. For example, resistance to trastuzumab may be conferred by the presence of a hetero-trimer of HER2, HER3 and insulin-like growth factor receptor (IGF-R).<sup>194</sup> Similarly, co-activation of c-Met and EGFR leads to resistance to EGFR-tyrosine kinase inhibition by, *e.g.* gefitinib.<sup>178</sup> However, a combination strategy using inhibitors of c-Met, EGFR, and platelet-derived growth factor receptor (PDGFR) is successful in reducing cell viability compared to single-agent treatment, as multiple co-activators are targeted.<sup>195</sup> Although this is a viable approach to chemoresistance, such a cocktail of drugs is unlikely to be tolerated in patients. The attractiveness of such networks is the ability to identify fragile points downstream of the activators which may be specifically targeted to overcome resistance. Recently HER3 was identified as an example of a fragile node, as well as a co-activator, in animal models.<sup>176</sup> However, the challenge in translation of these models for clinical use lies in the paucity of signalling data in humans. Networks constructed thus far rely on *in vitro* or biochemical analysis of tumour tissue. *In vivo* subcellular imaging techniques are likely to be the next step in providing the data required to further annotate these network maps for clinical use.

Conversely, signalling networks developed *in vitro* have the potential to identify novel imaging biomarkers for resistance or response, in order to optimise the use of targeted treatments (a more detailed description of this approach can be found in ref. 196). Table 1 illustrates a selection of the many and varied

1 biomarkers which may enter the clinical arena to optimise cancer therapy. However, the question of which biomarker should be imaged for a particular patient remains unanswered. For example, an EGFR-centred protein network has been

constructed and probed using small interfering RNA (siRNA), in order to highlight protein-protein interactions which may contribute to resistance or sensitivity to cancer therapy.<sup>33</sup> Several proteins of interest were identified as potential



1 regulators of response to treatment and therefore, may be  
2 candidates for development as imaging biomarkers. However,  
3 the complexity and robustness of protein signalling networks  
4 combined with the intrinsic error rate associated with siRNA  
5 screens means that these potential targets require thorough  
6 validation *in vivo* prior to translation to the clinic.

7 In addition to application to signalling networks, clinical  
8 imaging modalities may provide prognostic information  
9 concurrent with established prognostic tools, such as gene  
10 expression signatures, in order to construct a mathematical  
11 model for outcome prediction. The development of microarray-  
12 based gene expression signatures has enabled classification of  
13 tumour subtypes and association with clinical outcome, notably  
14 in breast cancer.<sup>19,197</sup> A 70-gene prognostic signature for lymph  
15 node negative breast cancer patients is reported to provide  
16 prognostic information independent of clinicopathological scores  
17 and with improved sensitivity and specificity for poor clinical  
18 outcome.<sup>198,199</sup> A validation study of the 70-gene signature  
19 reported 90% sensitivity for metastasis within 5 years, for  
20 example, with specificity of 42%.<sup>199</sup> The tumour heterogeneity  
21 observed within intrinsic molecular subtypes of breast cancer is  
22 beginning to be described as deregulated molecular pathways at  
23 the gene expression level.<sup>200</sup> Clinical imaging traits have been  
24 used to partially reconstruct gene expression variation between  
25 tumours.<sup>201</sup> Association maps between clinical imaging features  
26 and gene expression variation have been constructed for CT  
27 image traits in hepatocellular carcinoma<sup>202</sup> and MRI traits in  
28 glioblastoma multiforme,<sup>203</sup> suggesting that imaging traits can  
29 approximately predict gene expression variation between  
30 tumours.

## 6. Conclusions

31 We have summarised a variety of potential applications for  
32 molecular imaging, ranging from the nanometre to the whole  
33 body scale, in the optimisation of cancer therapy; *i.e.* choosing  
34 the right drug for the right patient at the right time. We have  
35 also discussed some of the challenges faced in the integration  
36 of molecular imaging into clinical practice.

37 Molecular imaging at a whole body level is unlikely  
38 to be possible using a single imaging modality. Tumour  
39 heterogeneity and a differential response to treatment  
40 represent a few of many characteristics influencing the  
41 eventual tumour phenotype, and thus, response to cancer

42 therapeutics. Whole body imaging is the only technique  
43 currently available for the study of both primary tumour  
44 and metastases, both at diagnosis and to monitor response  
45 to treatment, but may not provide vital information at the  
46 molecular level. Image-guided biopsy, co-registration of  
47 complementary imaging modalities and appropriate bio-  
48 marker choice may provide optimal risk stratification and  
49 help overcome these challenges. The issues of radiation  
50 dose and financial cost are yet to be fully addressed.  
51 Appropriate choice of imaging technology, the combination  
52 of modalities utilising ionising and non-ionising radiative  
53 sources and signal amplification may help alleviate some  
54 of the burden. Common quality assurance and control  
55 methods need to be developed in order to ensure a standard  
56 for imaging which may impact on treatment choice. This  
57 becomes increasingly difficult with rapidly evolving technology  
58 and whilst crossing national boundaries but is an area which  
59 requires future consideration.

60 Clinical outcome and response to treatment has a  
61 complex and multifaceted relationship to genotype and  
62 gene expression, dysregulation of signalling pathways  
63 *via* gene expression, protein activity, protein–protein  
64 interactions and tumour phenotypic traits. It may be  
65 possible to extract this data from patients using molecular  
66 imaging amongst other established techniques. However,  
67 the challenge is to integrate datasets from each observable  
68 level of variation, from genotype to tumour phenotype, in  
69 order to inform clinical management for the individual  
70 patient.

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## Notes and references

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82 **Fig. 3** Multiphoton FLIM measurements of the intermolecular FRET between ErbB1-GFP and ubiquitin-mRFP1. A. MCF cells were  
83 transiently transfected with EGFR-eGFP in the presence or absence of mRFP1-ubiquitin to assess EGFR ubiquitination using a FRET-by-  
84 FLIM assay. Cells were either EGF treated (100 ng ml<sup>-1</sup>, 30 min) or not. FRET between GFP and mRFP1 results in shortening of the fluorescence  
85 lifetime ( $\tau$ ) of GFP. FRET efficiency was calculated using the following equation in each pixel and averaged per each cell. FRET efficiency =  
86  $1 - \tau_{\text{da}}/\tau_{\text{control}}$ , where  $\tau_{\text{da}}$  is the fluorescence lifetime of cells co-expressing both EGFR-EGFP and mRFP1-ubiquitin and  $\tau_{\text{control}}$  is the mean  
87 EGFR-EGFP lifetime measured in the absence of acceptor. B. Endogenous EGFR ubiquitination is assessed in the breast cancer cell lines, HCC  
88 and MDA-MB-468. EGFR ubiquitination is increased in cells which highly degrade EGFR on EGF treatment (HCC) as opposed to those which  
89 degrade EGFR less readily (MDA-MB-468). This is shown by the pseudocolour fluorescence lifetime maps. Here, FRET was measured between  
90 Cy2 and m-RFP. (C) Translation of the above FRET-by-FLIM assays to dual fluorophore-labelled antibody assays for application to endogenous  
91 protein interactions in tumour tissue. These Figures show the initial assessment of FRET efficiency between anti-EGFR-Cy2 and anti-ubiquitin-  
92 Cy3 in A431 cancer cells (with the established methodologies being applied to patient-derived cancer tissues). Anti-ubiquitin-Cy3  
93 IgG concentration is at control level at 1  $\mu\text{g ml}^{-1}$ , whereby no ubiquitin staining is seen (epifluorescence Cy3 image), *versus* optimal  
94 concentration (10  $\mu\text{g ml}^{-1}$ ), whereby a reduction in fluorescence lifetime of Cy2 is seen on the pseudocolour lifetime images. White scale bars  
95 represent 5 microns.

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