

## MOLECULAR IMAGING – INTRODUCTION

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### Introduction

The primary role of biomedical imaging is in diagnostics, i.e. the characterization of a disease phenotype based on morphological or physiological readouts. Image quality is rated based on two criteria: spatial resolution and contrast-to-noise ratio. Image contrast and thereby information can be enhanced by administration of labels that either enhance the contrast in the image or generate a signal that otherwise would not be detectable. When combining a signal generating moiety (a reporter group) with a target-specific carrier (receptor ligand, enzyme substrate, antibody, oligonucleotide, cell) highly specific information on targeted molecular interactions can be obtained. Such target-specific or *molecular imaging* approaches raised considerable interest both from a diagnostic and therapeutic point-of-view<sup>1-3</sup>, and will become important tools for biomedical researchers. They provide tempo-spatial information on molecular processes in the intact organism, i.e. in the full biological context with all regulatory interactions in place. Molecular imaging techniques will therefore play a role for the validation for systems biological approaches.

As for structural and functional imaging a primary application area of molecular imaging will be in diagnostics. Tissue/organ structure and function can be considered a phenotype of a molecular program. Being able to visualize and quantify these molecular processes will therefore provide early information on pathologies, eventually in a pre-morbid state. Molecular information will certainly enhance the accuracy of diagnosis allowing for better therapy planning for the individual patient (personalized medicine). This implies also a key role for the development of novel therapies: Today, potential drug targets are discovered at a rapid pace using genomics and proteomics approaches. During target validation the expression levels and the function/dysfunction of a potential target have to be analyzed in the normal and diseased state, preferentially in the intact organism e.g. using molecular imaging approaches. Prior to entering a clinical development program - in particular when targeting chronic diseases with late clinical endpoints – imaging and in particular molecular imaging methods might be used to identify biomarkers for assessing treatment efficacy and demonstrating the validity of the therapy concept (proof-of-concept). Eventually such biomarkers, which might probe products of gene transcription, gene translation or metabolic alterations<sup>4</sup> may serve as surrogates for late onset structural or physiological effects of therapy. This is illustrated by a recent positron emission tomography (PET) study using [<sup>18</sup>F]-fluoro-deoxyglucose in patients suffering from gastrointestinal stromal tumors that underwent treatment with the Abelson tyrosine kinase inhibitor imatinib (Gleevec®). Within less than 24h after therapy onset the high glucose metabolic rate in the tumors was essentially reduced to undetectable levels; a significant reduction of tumor volume was only observed 6 months later<sup>5</sup>.

### Visualization of the expression of individual molecular targets

The possibility to image molecular events under in-vivo conditions lends itself to the study of potential drug targets. The direct approach is to use a labeled reporter ligand that directly

interacts with the target with high selectivity. The development of such probes addresses similar issues as the development of therapeutic drugs. 1) Molecular targets occur at low concentrations, in the extreme case only a few molecules per cell, rendering the sensitivity of the assay a critical issue. The detection method should be inherently sensitive and/or the reporter principle used should allow for significant amplification of the target specific signal; in other words, a single molecular target molecule should give rise to the signaling of a large number of reporter groups. 2) Pharmacokinetic (PK) properties of the reporter construct should be such that it reaches its target at high enough concentration to generate a signal. This involves passage of cellular membranes and sufficient long exposure time for the ligand-target interaction to occur. Ligand delivery is certainly an issue when large reporter groups such as paramagnetic complexes or nanoparticles are being used. 3) Cross-reactivity with other potential targets should be minimal to avoid confounding signals. 4) Any non-specifically bound fraction of the ligand should be rapidly eliminated to optimize the target signal-to-background contrast. 5) The ligand must be safe at the dose administered. Optimization studies for target-specific ligands are time consuming and expensive and therefore restricted to selected high value targets.

Direct target visualization using labeled reporter ligands is the classical molecular imaging application. Nuclear imaging and in particular positron emission tomography (PET) have been used for years to study receptor occupancy. A remarkable number of PET ligands have been developed to study neurotransmitter systems<sup>3, 6</sup>. With the development of optical imaging methods such as near-infrared fluorescence imaging (NIRF) target-specific probes have been developed using fluorescent dyes as reporter moiety. For example, receptor expression has been visualized using receptor ligands<sup>7</sup> or antibodies/antibody fragments labeled with the NIRF dye Cy5.5<sup>8</sup>. An attractive strategy to enhance signal-to-background ratio are probes that are 'silent' unless activated by the interaction with their molecular target. They are based on changes of the biophysical properties of the reporter group, for examples changes in the fluorescence yield (dequenching, fluorescence resonance energy transfer) or changes in their magnetic relaxation properties. Examples are the protease specific probes developed for NIRF imaging<sup>9</sup>, magnetic switches<sup>10</sup> or enzyme activatable paramagnetic complexes<sup>11</sup>.

Noninvasive direct target imaging approaches yield highly specific information on the expression of a drug target; yet, their applicability is limited by just this specificity – each target requiring its own specific probe. Therefore, indirect imaging techniques based on reporter genes have been developed as research tools allowing the study of the regulation and the temporo-spatial profile of the expression of the target gene. Reporter gene approaches are standard molecular biology tools to study gene expression in cultured cells and in whole organisms. Methods to non-invasively visualize gene expression in whole animals provide this information in a spatially and temporally resolved manner. Two strategies are being pursued: development of a genetically engineered mouse line expressing the reporter gene under the control of the target promoter and introduction of reporter gene cassette using a viral vector. Site-/ organ-specific reporter gene expression can be achieved by crossing a reporter mouse that ubiquitously expresses a reporter gene upon Cre-recombinase mediated recombination with animals that express Cre under the control of a site-specific promoter<sup>12, 13</sup>.

A considerable number of reporter gene systems has been developed and made available for non-invasive imaging applications. Bioluminescent and fluorescent systems are widely used in molecular and cellular biology and have been translated to in vivo imaging in a straightforward manner. The processing of D-luciferin by firefly luciferase yields a bioluminescence signal provided there are sufficiently high tissue levels of oxygen, ATP, and Mg<sup>2+</sup><sup>14</sup>. As there is no inherent bioluminescence in mammalian tissue a high signal-to-background ratio and thereby a high sensitivity is achieved. This approach is widely used by the imaging community. The green-

fluorescent protein (GFP) of the jellyfish *Aequorea victoria* and its enhanced variant (EGFP) is probably the most frequently used reporter system in biological research. Its in vivo applications are somewhat limited by the fact that light penetration into tissue at the EGFP wavelengths is limited. In addition, there is significant tissue auto-fluorescence in this spectral domain degrading the signal-to-background ratio. The use of red-shifted variants such as red fluorescent protein (RFP) or DsRed reduces these problems to some extent<sup>15</sup>. Detection of fluorescent proteins is not dependent on the administration of an exogenous ligand and its PK properties, a significant advantage.

PET reporter gene assays involve the expression of intracellular enzymes that modify and thereby trap a substrate labeled with a positron-emitting radionuclide or membrane receptors to which a labeled ligand binds. Herpes simplex virus-1 thymidine kinase (HSV1-TK) is the most widely used representative of the first type; a number of labeled thymidine analogs have been designed as PET substrate<sup>16, 17</sup>. HSV1-TK is sufficiently distinct from mammalian TK to allow for substrate specificity. Any membrane receptor, for which PET ligands exist, can in principle be used for a reporter gene assay as long as it is not naturally expressed by the target tissue. An example is the expression of dopamine-receptors in hepatocytes, which can be visualized using a labeled dopamine ligand that is commonly used for studies of the neurotransmitter system<sup>18</sup>.

MRI reporter gene products are either enzymes that alter the chemical structure of an exogenous paramagnetic substrate and hence its relaxation properties<sup>11</sup> or transporter proteins located in the cell membrane that lead to accumulation of an exogenous paramagnetic or superparamagnetic ligand in the cell<sup>19</sup>. PK properties of the reporter moiety largely govern the applicability of such reporter constructs, a significant limitation of the approach. More recently, a reporter system has been developed, which is independent of an exogenous ligand; expression of metalloproteins of the ferritin family leads to the sequestration of endogenous iron into superparamagnetic clusters in genetically engineered cells/tissue<sup>20</sup>.

An alternative strategy can be pursued when studying receptor activation in the CNS, the use physiological coupling as amplifier of molecular interactions. Assuming that drug-receptor interaction will lead to activation or deactivation of specific brain areas, functional MRI (fMRI) techniques can be used to monitor the functional consequences due to neuroactive drugs. This has been demonstrated for the dopamine transporter<sup>21</sup>, GABA<sub>A</sub> receptors<sup>22</sup> and opiate receptors<sup>23</sup>. Although not being a molecular imaging approach *per se*, the fMRI signals combined with pharmacological stimulation, i.e. by administration of a CNS active drug, constitutes an attractive approach providing relevant information on the drug-target interaction with high spatial and temporal resolution, which is translatable in a straightforward manner into the clinics.

### **Signal transduction pathways**

Direct visualization of target expression is complemented by imaging of target function, i.e. of downstream processes activated by ligand-target interaction. In fact, the assessment of the functional response to a drug target-interaction, e.g. the flux through a signal transduction pathway, can be considered more relevant than the visualization of the target expression or the receptor occupancy by the drug *per se*. Visualization of individual molecular players in a signaling cascade is based on identical assays as those described for target imaging.

Elegant assays have been developed for visualization of protein-protein interactions, all of them requiring genetic engineering of target cells. The design must be such that a reporter moiety is only expressed or activated when the two interacting proteins are in close proximity. Four approaches have been described in vitro and three of them have been translated to allow for studies in intact animal studies: fluorescence resonance energy transfer<sup>24</sup>, the 2-hybrid system<sup>25</sup>, the protein fragment complementation assay<sup>26</sup>, and protein splicing<sup>27</sup>. It is beyond the scope of

the article to describe the individual approaches (see e.g. <sup>3</sup>). Nevertheless, it is to be anticipated that such assays will become major tools for the study of intracellular signaling in the intact organism, i.e. in the full biological context.

### Cell labeling

Many pathological events involve the migration of cells such as the infiltration of monocytes and lymphocytes to sites of inflammation or the formation of metastatic lesions in cancer. On the other hand, cell based therapies are playing an ever increasing role in modern medicine. Techniques that allow visualization of cell migration in vivo are therefore of high relevance both as diagnostic tools and for biomedical research.

Again different strategies for cell labeling can be pursued: passive labeling via internalization of a reporter moiety, targeting of cell-specific membrane receptors, or stable transfection of cells using reporter gene constructs (cell marking). For example, monocytes can be effectively labeled in situ by exploiting their phagocytotic properties: superparamagnetic or fluorescent nanoparticles will be rapidly internalized. The approach has been used to study a wide range of inflammatory diseases including neuroinflammation <sup>28-30</sup> or atherosclerotic disease <sup>31</sup>. All other cell types have to be labeled in vitro and re-administered to the organism. This is achieved using standard cell biological techniques, e.g. by co-administration of the reporter moiety with transfection agents. This approach has been used to monitor the migration of labeled stem and progenitor cells in models of CNS trauma <sup>32,33</sup>.

Gene marking of cells by stable transfection with reporter genes is a widely used concept in cell biology and meanwhile also in non-invasive imaging. In addition the use of reporter genes yields a cell viability assay: only living cells will express the reporter gene. Examples include the visualization of the migration of neural progenitor cells in a murine cerebral ischemia model <sup>34</sup> or of the formation of micro-metastases in bone marrow <sup>35</sup>. Similarly, fluorescence techniques have been applied to visualize cancer cells that stably express GFP to monitor tumor and metastasis formation in nude mice <sup>36</sup> or to study gastro-intestinal bacterial infection following intra-gastric administration of GFP expressing *Escherichia coli* <sup>37</sup>.

**Table 1:** Comparison of molecular animal imaging techniques with regard to sensitivity, specificity (discrimination of specific interaction from background), spatial resolution, time resolution (foreseeable throughput), quantification characteristics (relative and absolute concentrations of label) and translatability into clinics. (+: low, ++: medium, +++: high)

Method	Sensitivity	Specificity	Spatial resolution	Time resolution	Quantification	Translatability into clinics
MRI	+	+	+++	+	++	+++
Optical <sup>1)</sup>	+++ <sup>2)</sup>	+++ <sup>3)</sup>	+	++	+, ++ <sup>4)</sup>	+
Nuclear	+++	+++	++	+	+++	+++

1) Fluorescence and bioluminescence techniques

2) assuming high concentration of label (dye); not substrate limited

3) specificity high for bioluminescence, stronger background interference for fluorescence methods

4) better quantification to be expected for tomographic systems

### **Potential/limitations of molecular imaging: Comparison of methods**

The ideal molecular imaging technique provides high temporo-spatial resolution, high sensitivity, high specificity, is quantifiable and safe. In addition, it must be non-invasive for being translatable into the clinical setting. The ideal technology fulfilling all these criteria does not exist and compromises have to be searched for. An overview comparing the MRI, optical and nuclear imaging methods is given in Table 1.

Spatial resolution is limited either by physical limitations of the specific technology (PET, optical imaging) or by practical aspects such as unacceptable measurement times (MRI). The detection of molecular events occurring with low incidence, i.e. at low concentration, requires high sensitivity of the in-vivo detection method. For animal studies nuclear and optical imaging approaches are comparable, as the inherently lower sensitivity of the latter can be compensated by using high amounts of dyes. MRI is an inherently insensitive technique, a significant limitation with regard to target-specific imaging approaches.

Specificity is defined as ratio of the target specific signal in relation to the background signal due to unspecific binding. In the case of bioluminescence imaging specificity is inherently excellent due to the absence of a bioluminescence background signal. For the other modalities, specific signals can be enhanced by suitable amplification of the label concentration/signal at the target site. Approaches are ligand trapping and ligand activation by the target. Despite significant research with regard to optimizing the sensitivity of the reporter probes themselves (improved fluorescence properties, improved magnetic relaxivity), high signal amplification improving both sensitivity and specificity is critical and improved amplification strategies need to be developed.

Applications in biomedical research depend on the possibility to derive absolute or relative quantitative data from the imaging data sets. This is relatively straightforward for nuclear imaging: the amount of radioactivity detected is directly proportional to the amount of radionuclide present assuming proper corrections for physical and instrumental limitations have been made. Sophisticated models have been developed to account for PK aspects of ligand delivery and for the lifetime of the tracers. In MRI the correlation between the signal intensity and the amount of specific label present is non-linear and determined by several physical parameters making absolute quantification difficult. Nevertheless, reliable determination of relative concentrations is feasible. In optical imaging the signal is heavily surface weighted due to scattering and absorption of light by biological tissue. Relative concentration measures can only be compared assuming constant geometry. It is anticipated that the introduction of tomographic approaches based on fluorescence readouts will improve the quantitative aspects of optical imaging.

Probe delivery to the target site is a critical factor in molecular imaging. For fluorescence imaging and MRI requiring the administration of an exogenous label, the reporter groups are, in general, bulky molecules, which have a dominant influence on the PK properties of the ligand-reporter construct and are unable to cross the cell-membrane. In fact, the predominant number of NIRF and MRI studies to date has targeted endo-vascular or extracellular targets. Several concepts have been developed to enhance cell penetration of such reporter groups<sup>32, 38, 39</sup>. Target delivery is less a problem for PET: inserting a radionuclide via isotopic substitution into a small molecular receptor ligand does not affect its PK properties. If the parent molecule reaches its target the imaging ligand will do as well. Most of the reporter gene assays using optical or PET reporters allow the study of intracellular targets.

Today, in-vivo imaging has become indispensable in biomedical research. The development of target-specific or molecular imaging techniques has certainly added a new dimension: Structural and functional information can now be complemented by data on molecular biology collected in the intact organism. The success of molecular imaging applications will critically depend on

probe development. On the technology side developments will strive for multimodal imaging approaches, i.e. the combination of complimentary techniques, and the tools for quantitative data analysis, i.e. the determination of concentrations, activities, and flux rates instead of signal intensities.

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