Optical imaging for the new grammar of drug discovery

BY THOMAS KRUCKER* AND BRITTO S. SANDANARAJ

Global Imaging Group, Novartis Institutes for BioMedical Research, Cambridge, MA 02139, USA

Optical technologies used in biomedical research have undergone tremendous development in the last decade and enabled important insight into biochemical, cellular and physiological phenomena at the microscopic and macroscopic level. Historically in drug discovery, to increase throughput in screening, or increase efficiency through automation of image acquisition and analysis in pathology, efforts in imaging were focused on the reengineering of established microscopy solutions. However, with the emergence of the new grammar for drug discovery, other requirements and expectations have created unique opportunities for optical imaging. The new grammar of drug discovery provides rules for translating the wealth of genomic and proteomic information into targeted medicines with a focus on complex interactions of proteins. This paradigm shift requires highly specific and quantitative imaging at the molecular level with tools that can be used in cellular assays, animals and finally translated into patients. The development of fluorescent targeted and activatable ‘smart’ probes, fluorescent proteins and new reporter gene systems as functional and dynamic markers of molecular events in vitro and in vivo is therefore playing a pivotal role. An enabling optical imaging platform will combine optical hardware refinement with a strong emphasis on creating and validating highly specific chemical and biological tools.

Keywords: diagnostics; molecular imaging; disease; multi-spectral imaging; smart probes; probe design

1. Introduction

Optical molecular imaging promises to have a substantial impact in drug discovery as the development of new hardware, reporter gene technology, and novel targeted and activatable (‘smart’) probes enables the acquisition of unique information at the microscopic and macroscopic level. Combined these optical tools can be used at different stages of the drug discovery process to understand disease mechanisms at the molecular level, and to design, characterize and optimize therapeutic strategies (e.g. measure target expression levels or receptor

*Author for correspondence (thomas.krucker@novartis.com).

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occupancy, proof of mechanism of action or the pharmacodynamics (PD)/efficacy of drug candidates). A multi-modality approach of different optical technologies can in sequence or in parallel provide information at the molecular level from cells, tissues, organs and whole organisms. Notably, more recent developments promise that in the near future, optical imaging may also be routinely applied to diagnostic and therapeutic applications in clinical practice [1–5]. Optical molecular imaging has the potential to significantly and in unique ways contribute to the decision making and accelerate the time-consuming process of drug discovery.

2. The new grammar of drug discovery

Typically medical textbooks are organized by organ systems, and diseases are classified by their pathology or physiology. However, to understand the heterogeneity of common diseases guiding the target identification for drug discovery, a molecular definition must underlie the clinical manifestations. A time and location-dependent description of gene transcription profiles alone would be chaotic. Additional incorporation and understanding are needed for a ‘grammar’ that could be applied from the discovery of a drug target, to the testing in animal models, and lastly to the treatment of patients. As defined by Fishman & Porter [6], the grammar would be imbedded in a biological system as a set of rules for translating reliably and systematically gene products into medicines. Individual genes and gene products are abundant (greater than 25 000); however, the complex interactions of these proteins form about 200 molecular signalling pathways that are well conserved throughout most species, providing the keystone for the new grammar. Modulating sensitive pathway nodes (involved in one or several diseases) with targeted drugs would deliver the ‘magic bullets’. The identification and choice of a gene product of clinical relevance, the choice of targets, have to be convincingly related to disease, and the validation is typically a combination of studies in epidemiology and disease physiology, and from the results of research with animal models. To define the role of pathways in complex disease, molecular imaging using optical technologies offers a unique tool that enables the analysis of complex signalling networks at the level of individual pathway nodes. Particularly, in vivo optical imaging determines the activity specific to a particular cell type, dynamic feedback mechanisms, the kinetics of signalling and their state of activation in disease. With these promises come high expectations for optical molecular imaging and the need for the development of highly specific imaging tools.

3. Optical molecular technologies used in drug discovery

(a) Targeted imaging

The choice of the imaging method used for in vivo visualization and quantification of molecular processes is mainly driven by the strengths and weaknesses of the modality. Magnetic resonance imaging (MRI) and computed tomography (CT) offer relatively high spatial resolution, deep tissue penetration and excellent soft tissue contrast, but a major limitation is the poor sensitivity, limiting their role...
in molecular imaging [7]. The micromolar amounts of contrast agents required for signal generation are several orders of magnitude higher than molecular targets normally present at nano- or sub-nanomolar concentrations.

Optical imaging technologies are particularly suited for the field of molecular imaging. They are well established in the preclinical arena (in vitro and in vivo), but somewhat limited in clinical application because of the absorption of light in the tissue. Light emission can be induced by enzymatic reactions of a substrate with cells or organisms transfected with bioluminescent endogenous reporters, e.g. luciferase from the North American firefly [8]. Reporter gene assays have been demonstrated to yield fundamental biological information related to molecular pathways or targeted drug action. Signal is detected either by photomultiplier tubes or cooled charge-coupled devices, and different strategies are used to minimize endogenous background signal [9,10]. Using fluorescence imaging, exogenous fluorochromes or endogenous reporters (e.g. fluorescent proteins) are excited by a light source (either lasers or halogen lamps), and the emission of the fluorescent reporter is then detected. Near-infrared (IR) light is particularly suited for in vivo imaging as tissue has its lowest absorbance in this region (650–900 nm), and scattering is the most significant attenuation factor. Current developments on the hardware and reconstruction algorithms will improve signal localization and quantification by introducing fluorescence tomography approaches, possibly combined with other modalities [11,12]. The development of optoacoustic tomography is going to allow imaging of physiological and molecular markers at higher resolution [12]. These innovations will further advance the scope of imaging in the area of medicine and biology and certainly have an impact on the drug discovery process.

Reporter molecules for optical imaging consist of a (near-IR) fluorescent dye, which can be coupled to target-specific ligands/carriers analogously to radiolabelling methods (see below). In addition, fluorescence detection allows researchers to design smart sensors based on fluorescence quenching mechanisms [13], which are not detectable in their native state, but are activated by interaction with their target (e.g. protease sensors). Recent efforts in developing non-invasive (e.g. breast imaging) and minimally invasive imaging devices promise the translation of such tools into clinic [5]. Live cell and intravital microscopy (IVM) are other powerful optical imaging technologies, which are closing the gap between in vitro and in vivo assays by providing molecular information in high resolution [14,15]. Ideally, a comprehensive preclinical imaging platform consists of live cell microscopy, IVM and macroscopic imaging for whole animal imaging (fluorescence and bioluminescence). These tools enable the characterization and profiling of promising bioactive compounds (especially biologics such as antibody formats, peptides and proteins) on the molecular level in a relevant environment. A promising avenue to translation into the clinical arena would be by either directly using fluorescent probes or by substituting the fluorophore with a radioisotope for positron emission tomography (PET) imaging.

(b) From bench to bedside

Molecular imaging promises to enhance both discovery and development processes for devising more effective interventions. However, to accelerate biomedical research and development, multi-disciplinary skills and expertise
are required, which depend on a comprehensive knowledge of the biological systems, in addition to the physics, chemistry and computer science that underlie technologies. In order to accelerate and enhance all phases, a framework is needed for selecting, guiding and monitoring disease. As therapies become directed at specific biochemical pathways, tailoring treatments to the biochemical status of individual patients will be necessary. Optical imaging should be able to provide means to determine the type and required dose of drug for successful treatment. Imaging can guide targeted therapy by conceiving an initial treatment regimen, suggesting a change in therapeutic plan depending on patient response. It will also allow monitoring of side effects during treatment, and mapping drug delivery to diseased and normal tissues with the goal to minimize off-target toxicity in normal tissue. Atherosclerotic plaques are recognized as one of the major biomarkers in the inherently complex cardiovascular disease and the best predictors of imminent heart attack and stroke. However, to enable diagnosis, determination of disease progression and effects of therapeutic intervention, the direct assessment of atherosclerotic plaques is an unresolved challenge in clinical practice. Current imaging solutions are unable to provide the necessary molecular and spatial resolution [16]. Intravascular optical coherence tomography (OCT) has the potential to overcome these major limitations [17]. Initial developments have shown the feasibility of assessing arterial wall pathology in vivo. Atherosclerotic plaques can be diagnosed with high accuracy, including measurement of the thickness of fibrous caps and reporting the risk of rupture [18]. Such detail would enable efficacy assessments and monitoring the course of a therapeutic intervention at the necessary spatial resolution. Nonetheless, in the future, molecular information about the drug target and the modulation of associated molecular pathways will be necessary and only possible in combination with highly specific optical probes. A recent presentation reported about the development of such optical molecular reporters for cathepsin proteases and \( \alpha v \beta 3 \) integrin, and illustrated the preclinical feasibility of such an approach in assessing the efficacy of therapeutic intervention using Ezetimibe [19]. Coalescing optical instruments and targeted probes will potentially allow significantly more efficient clinical trial design for such drugs, starting with efficient proof of concept clinical studies and reduced numbers of patients enrolled in subsequent phases because of rigorous stratification of patients and a robust biomarker that will allow the direct monitoring of therapeutic interventions. Optical molecular imaging is offering unique and exclusive capabilities for unravelling the complexity of biological systems, reducing longstanding barriers in basic medical science, and providing a powerful new tool to improve drug discovery, development and the treatment of patients.

\[ (c) \text{Future optical technologies in drug discovery} \]

One of the major technical advances has been geared towards improving spatial and temporal resolution of the optical signal. Super-resolution microscopy techniques such as stimulated emission depletion microscopy (STED), stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM) allow, in some cases, sub-20 nm resolution in all three dimensions [20]. The next step will be high-speed, three-dimensional super-resolution fluorescence imaging, which will allow these techniques to be used to...
image the dynamic cellular environment at extraordinary levels of detail. Such developments will be crucial in unravelling molecular mechanisms underlying diseases and revealing the mechanism of new therapeutics. In the in vivo field of preclinical macroscopic optical imaging, improvement of depth resolution, sensitivity (quantitation) and dynamic continuous imaging will be at the forefront of technical evolutions. In the near future, such devices will be commercially available using hybrid systems with combined modalities (optical with CT or MRI) [11] and photoacoustics [21]. However, the foremost need and most probably the biggest hurdle for successful implementation into drug discovery of such new technologies will be the development of novel highly targeted imaging probes and new imaging probe technologies. Once such probes are available, also translation into the clinic will become more attractive and facilitate the development and distribution of new clinical optical devices that will assist diagnosis and surgery [22,23].

4. Endogenous and exogenous imaging probe technology

Reporter gene technology can be broadly classified into enzymatic reporter systems (β-galactosidase, luciferase and β-lactamase) and fluorescent proteins [24–26]. In an enzymatic reporter system, photons are generated through chemical reactions and therefore called bioluminescence, whereas fluorescent proteins emitting light when excited by a photon of particular energy refers to fluorescence. The main disadvantage of the fluorescent proteins is the lack of signal amplification, whereas enzymatic reporters will catalyse the turnover of multiple substrate molecules, allowing much lower level of gene expression to be detected. However, enzymatic reporters do require the administration of exogenous small molecules (e.g. luciferin in the case of the firefly luciferase). This technology was exploited to decipher some of the fundamental biological processes, such as gene transcription [27], translation, protein–protein interaction [28], protein–substrate interaction [29] and signal transduction [30]. This technology has been widely used in drug discovery for target discovery [31], target validation [32], lead optimization [33], biodistribution [34], pharmacokinetics (PK) and PD properties.

5. Exogenous probe technology or synthetic fluorophores

(a) Probe synthesis and validation

The development of optical contrast agents involves validation of new probes at several stages, which include design and synthesis, in vitro, cellular and in vivo studies. Such developments are very similar to a drug discovery effort, but can often be combined or are ideally run in parallel. The first step in the development of an optical imaging agent is the design of the molecular reporter to answer a specific biological question followed by validation at the in vitro stage, i.e. studying the selectivity of the probe towards the target protein. As most of the drug targets are intracellular, probes are typically designed in such a way that they can cross the plasma membrane of a cell and more importantly, be delivered to the right organelle or location where the target is present.
(e.g. endosome, lysosome, golgi apparatus, endoplasmic reticulum, etc.). In addition, for in vivo applications probes should have optimal biodistribution/PK properties.

(b) Substrate-based reporters

The concept of a substrate-based reporter refers to a small-molecule fluorescent probe whose recognition and cleavage moiety mimics the binding site of a true substrate (peptide/protein substrate) in the native biological system. The fluorogenic substrate reporter should satisfy three important criteria. The substrate should contain (i) a cleavable bond that can be hydrolysed by the enzyme, (ii) the recognition element that can take the substrate into the active site of the enzyme, and (iii) the fluorescent reporter should be activated instantaneously after the hydrolysis of the cleavable bond.

The activation of a fluorescent signal can happen through two different mechanisms that either depend on a change in the redox potential of the fluorophore after cleavage of the peptide, or the separation of fluorophore/quencher pair that abolishes fluorescence resonance energy transfer (FRET), photoinduced electron transfer or proximity-induced fluorescence quenching. For example, the FRET concept has been widely used to create both endogenous and exogenous fluorescent reporters. In a classical example, Jones et al. designed a reporter for caspase-3. This reporter consists of blue fluorescent protein (BFP) separated by a peptide sequence specific for caspase-3 attached to green fluorescent protein (GFP). This assay is widely applied in high-throughput assays for the screening of anti-cancer agents [35]. The Weissleder group exploited the concept of proximity-induced fluorescent quenching to create protease-sensitive smart probes, which can be used for in vivo macroscopic and microscopic imaging applications [36].

(c) Latent fluorescent reporters

Traditional fluorophores are inherently fluorescent; consequently, bulk fluorescence can obscure valuable information. Therefore, the idea of converting a fluorescent molecule to a latent fluorophore has gained much attention in recent years [37,38]. This is usually done by coupling a fluorescent reporter to a chemical moiety that changes the fluorescence properties (usually fluorescence quenching) of the parent molecule. The chemical moiety is designed in such a way that it can be cleaved by specific enzymes/proteins present in the biological milieu. Upon enzyme cleavage, the fluorophore is brought back to its fluorescing state. The advantage of this method is the minimal change in the structure of the probe with respect to the original peptide/protein recognition site. The disadvantage is the direct coupling of the fluorophore with the cleavable amide bond that might decrease the binding affinity of the substrate towards the target enzyme. In particular instances [39], systematic variation of the fluorophore linkage resulted in optimized signal-to-noise ratio and improved specificity to caspase-3. For example, Kamiya and co-workers designed a probe for β-galactosidase using this concept. They have successfully used this probe for tumour imaging in vivo [40].

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(d) **Substrate reporter based on fluorescence resonance energy transfer mechanism**

Bullok & Piwnica-Worms [41] developed a near-IR probe for caspase-3 to image apoptosis and showed successful application *in vitro* and *in vivo*. The C-terminal side of caspase-3 substrate was tagged with Alexa Fluor 647 as the energy donor, and the quencher QSY21 as the energy acceptor was placed at the N-terminal side of the substrate. In the absence of caspase-3, fluorescence of Alexa Fluor 647 is quenched by QSY21 through FRET mechanism. Addition of caspase-3 results in the cleavage of the amide bond next to aspartic acid that leads to fluorescence activation. *In vitro* profiling studies showed the $K_m$ value of caspase-3 for this probe was six times higher than caspase-7 and 16 times higher than caspase-6. Cleavage of the substrate by initiator caspases was not observed. This probe was then successfully used to image apoptosis in cells and *in vivo*.

(e) **Activity-based fluorescent probes to image protease activity**

Activity-based fluorescent probes (ABFPs) take molecular imaging to the highest level of specificity [42–45]. They are designed to detect and report the presence of only the activated target or pathway node. ABFPs have been widely used in the field of proteomics and, to some extent, in molecular imaging [46]. They are small molecules that can react with a catalytic nucleophile present inside the active site of a target enzyme. ABFPs share three core structural elements: a reactive functional group, a peptide scaffold for recognition or binding and a fluorescent reporter. The active site modification of the enzyme by the small molecule is reported by the change in the fluorescence signal. Since the reaction is based on a specific mechanism and involves the participation of active enzyme, the extent of active site modification serves as an indirect readout of activity levels within a given sample. The specificity of the probe molecules towards a particular enzyme can be modulated by engineering the reactive functional groups and the scaffolds that carry the reporter tag.

(f) **Non-invasive imaging of cathepsin activity**

Upregulation of cysteine cathepsin is associated with many diseases, including cancer, atherosclerosis, inflammation and Alzheimer’s disease [47,48]. Bogyo and co-workers [47] reported the use of ABFPs for monitoring cathepsin activity in an *in vivo* tumour model. The probes showed punctuate labelling of lysosomal compartments that overlapped with acidotropic lysosomal marker. Pre-treatment of cells with the cathepsin inhibitor JPM-OEt abolished the staining establishing its specificity. The successful results from these *in vitro* validation studies laid important groundwork for the *in vivo* characterization of the probes. Initial time points of the *in vivo* experiment revealed that the specific probes and the control probes circulated uniformly resulting in high, unspecific background signal. Accumulation in the tumour region was observed only for the probes with the warhead acyloxymethyl ketone (AOMK) group. By contrast, control probes were rapidly cleared and no fluorescent signal was observed beyond 5h post-injection. One of the main advantages of the activity-based fluorescent method is the back tracking of *in vivo* data by additional biochemical profiling of tissue homogenates collected from animals treated with ABFPs. Gel electrophoresis
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studies of tissue lysate obtained from in vivo experiments showed labelling of three predominant proteins in the size range 20–30 kDa. The 30 kDa protein was confirmed to be cathepsin-B, and the 23 kDa protein was identified as the heavy chain form of cathepsin-L by immunoprecipitation. In addition, the utility of this method to image the efficacy of small-molecule inhibitors was explored. Mice were treated for 5 days with a broad-spectrum cathepsin inhibitor prior to imaging in order to completely reduce the activity of cathepsins. Quantitation of total tumour fluorescence indicated that signal was significantly reduced by 60–80% in treated mice relative to control mice at the 10 h time point. Importantly, subsequent quantitation of the biochemical gel data indicated that labelling of specific cysteine cathepsins was reduced by 50–80%, in tissues from the treated mice, which correlates well with the observed imaging data.

(g) Imaging apoptosis

Programmed cell death (apoptosis) plays a crucial role in both normal and pathological processes. Improper apoptosis is associated with several diseases including Alzheimer's disease [49], ischaemia [50] and cancer [51]. Apoptosis occurs in different distinctive steps that include disruption of transmembrane potential of mitochondria, release of cytochrome c from mitochondria to cytosol, caspase activation (conversion of inactive enzyme to an active enzyme), chromatin condensation, DNA fragmentation and membrane disintegration. Caspases are considered key players in the early event of the apoptotic process and are highly specific. One of the commonly used apoptosis probes is fluorescently labelled annexin V, a protein with high affinity for phosphatidylserine that is exposed during the late stage of apoptosis [52]. The main limitation of the annexin V method is given by the fact that it cannot differentiate between apoptotic cells and necrotic cells. In order to increase the selectivity of a probe towards caspase-3, Edgington et al. [53] engineered a probe where the sequence E-P-D was chosen based on in vitro profiling results obtained from various custom-made caspase-3 substrates. This E-P-D sequence is more selective compared with the commonly used caspase-3 substrate D-E-V-D, which is known to react with similar classes of proteins such as legumain and cathepsins. Near-IR fluorophore Cy5 was attached to N-terminal residue glutamic acid (Glu), and the C-terminal residue aspartic acid (Asp) was converted to AOMK group, which is very specific for cysteine proteases. In order to increase the cell permeability of the probe, a Tat peptide sequence was attached to the N-terminal side of the Cy5-Glu-Pro-Asp-AOMK probe. The control probes had no reactive functional group. The ability to label caspase-3 in intact cells was successfully tested upon apoptosis induction of Jurkat cells with anti-Fas antibodies. The addition of Tat peptide resulted in a substantial increase in the labelling attributed to the uptake via the endo-lysosomal route before release into the cytosol. In vivo, caspase-3 activity in CD4+ CD8+ thymocytes was observed at 6 h after treatment with dexamethasone, peaking at 12 h post-treatment and then sharply dropping to background levels at 24 h after injection. Post-processing of the tissue showed that labelled caspase correlated with the overall signal observed for thymi in the imaging system, suggesting that fluorescence observed in whole organs can be used as a direct readout of total probe-labelled proteases.

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(h) Fluorescent nanoprobes as a biomarker for increased vascular permeability

The use of optical methods to assess vascular leakage is an attractive imaging-based biomarker in disease models [54,55]. As a proof of concept, we have shown that optical imaging combined with fluorescent nanoprobes can be used effectively as a functional biomarker for the identification of increased vascular leakage [55]. The versatility of this method is demonstrated by using this technique for diagnosis and monitoring of two different disease conditions, where vascular leakage exists. In vivo studies in mice bearing PC3 tumours revealed that at 6 h after IV administration of Cy5.5-labelled liposomes, particles were distributed uniformly throughout the body. However, at the 24 h time point, the tumour could be differentiated because of the enhanced fluorescence signal from the tumour region. At 48 h after probe administration, the fluorescence signal intensity from the tumour area further increased, but the fluorescence signal from other parts of the body remained the same as at previous time points. The fluorescence signal at 72 h decreased considerably, returning at 96 h back to baseline. The fluorescence signal in non-tumour area was significantly lower than that in the tumour and remained at about the same level during the entire study period. Overall, these results show an accumulation of the probe in the tumour owing to the presence of leaky vessels in this area.

Another application is a murine model of antigen-induced arthritis, in which the induced leakage of the vasculature is colocalized with the inflammatory response. The antigen was injected on day 0 into one knee while the contralateral knee served as control. Liposomes labelled with indocyanine green were administered intravenously at day 4 after antigen treatment. There was no preferential accumulation of particles in the arthritic joints at initial time points. However, at 24 h after probe administration, a twofold increase in fluorescence signal was observed in arthritic compared with control, non-arthritic joints. These observations suggest a passive accumulation of the probe in arthritic joints because of the leaky vasculature. To establish whether the fluorescent nanoparticles are specific and sensitive enough for assessment of treatments, the effects of a corticosteroid, dexamethasone, on vascular leakiness were investigated. Animals were treated orally with vehicle or dexamethasone at a dose of 0.3 mg kg$^{-1}$ from day 0 to 7. Nanoprobes were injected IV on day 4. In dexamethasone-treated mice, the fluorescence signals in arthritic joints were similar to those in contralateral, control joints. By contrast, in vehicle-treated animals, the signals from the arthritic joints were twofold higher than those from non-arthritic joints. This approach can be used as a highly specific tool to screen the efficacy of anti-inflammatory compounds in a preclinical setting. Because of the components and the clinically approved fluorophore, it also provides the concept for equivalent studies in arthritis patients.

(i) Role of optical molecular imaging in the development of biologics

The role of optical molecular imaging in the discovery of biologics as therapeutics has been rapidly increasing in recent years [56,57]. Antibodies or antibody fragments are used as tracers in molecular imaging applications because of their high affinity and selectivity to extracellular targets. These agents are primarily used to measure the PK and biodistribution [58] of a biopharmaceutical drug by using labelled derivatives and study the target of corresponding
therapeutics non-invasively (e.g. target expression levels or receptor occupancy to prove the mechanism of action or the PD/efficacy of the corresponding drug). Given their relatively large size, biologics can be labelled with fluorescent dyes with minimal changes to their properties and these conjugates can be used in subsequent optical imaging studies. It can be imagined that fluorescence imaging might serve as an economical and rapid screening tool delivering decision-relevant information to justify a further time-consuming and costly nuclear tracer development for PET imaging used in clinical applications [59].

(j) Activatable ‘smart’ and targeted probes with high specificity

Weissleder and co-workers [36] introduced the concept of activatable ‘smart’ probes. The concept is simple but powerful. Since these agents are polymeric in nature, they also have very good PK and biodistribution properties, which are crucial for in vivo imaging application. The modular design allows one to create probes for different proteases. To date, several smart probes are designed for various proteases which include cathepsins [36], matrix metalloproteases [60], thrombin [61] and plasminogen activator [62,63]. The success of these probes in the preclinical research setting led to the first application of such agents in the clinic. Hintersteiner et al. [64] developed a near-IR fluorescent probe for beta-amyloid plaques, a hallmark of Alzheimer’s disease. Different therapeutic strategies are targeting the prevention and elimination of beta-amyloid formation and aggregation [65]. This highly targeted probe has a binding affinity of 0.2 μM towards amyloid plaques and crosses the blood–brain barrier. Using APP23 transgenic mice as a model for Alzheimer’s disease, it was demonstrated that this probe can detect, non-invasively, amyloid and amyloid plaques and can be used to test anti-amyloidogenic therapies [11,64].

6. Conclusion and outlook

From a drug discovery perspective, the development of commercially available, high-quality optical microscopic and macroscopic imaging equipment for in vitro and in vivo applications has been a significant breakthrough in the last decade. Particularly, systems offering complete solutions packaging hardware and software that enable high-throughput, high-reproducibility and simplified image analysis without compromising flexibility (and to some degree customization) are attractive for an imaging platform in a drug discovery environment. However, the initial excitement has been somewhat hampered by the realization that engineering solutions often fail to directly translate into tangible successes in drug discovery because the critical questions are driven by the biology, and applications have to be developed and rigorously validated before they are considered relevant and can be used. Nevertheless, offering ‘push-button’ options may facilitate the commercialization and the access to optical technologies, but carries the danger that the technology is not fully understood and creates nonsensical data.

As important and acting as the enabler of the technological solutions in the area of imaging in drug discovery has been the creation of specific fluorophores and fluorescent proteins. The discovery of GFP and developments that led to its use as a tool as a reporter gene assay were recognized with a Nobel Prize in 2008 [66]. Although not ideal for non-invasive optical imaging, the concept has

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led to the development of an extended colour palette beyond green, with proteins such as mCherry, tdTomato and many others [67] with emission spectra closer to near-IR suitable for non-invasive imaging.

The development of optical probes is driven by the type of biological question to be addressed. The design, synthesis and validation of optical probes are time-consuming and challenging. New approaches like ABFPs and supramolecular/nanoparticle-based imaging probes are being actively pursued and offer huge advantage over small-molecule imaging probes. The next decade will see an explosion of this novel approach and to allow success, it will be necessary to engage early into rigorous validation.

Another area of optical fluorescence imaging with very high potential of being successfully applied in drug discovery is the development of biologics. Tools are being developed that allow assessment of the PK and biodistribution of these biomacromolecules and enable the investigation of their corresponding targets in vivo. On the other hand, imaging agents based on biologics might be developed as valuable imaging biomarkers supporting diagnosis. Knowledge of the dynamics of biological events is crucial for the successful validation of probes at both the cellular and in vivo level.

Despite the fact that the limited tissue penetration of light restricts fluorescence imaging applications mainly to small rodents, human studies using the technique have been reported. Applications include the quantification of oxy- and deoxy-haemoglobin [68], the detection and characterization of breast cancer [69,70], arthritis in finger joints [71] and the detection of functional activity in the brain [72]. A theoretical study of the feasibility and limitation of using optical probes in human organs has been presented by Ntziachristos et al. [73]. These examples illustrate the clinical potential of optical imaging. Nevertheless, the design, validation and clinical approval of novel, more selective tracers will represent one of the biggest challenges.

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