Pre-clinical optical imaging and MRI for drug development in Alzheimer’s disease

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Optical and magnetic resonance imaging have the potential to be complementary non-invasive imaging modalities. Yet without advances in imaging technologies and contrast agents both have shortcomings that cannot be ignored. In this review we demonstrate the pre-clinical use of the two imaging techniques in Alzheimer’s disease, including examples from recent applications and discuss what is needed to improve their applicability for drug discovery.

Introduction

Alzheimer’s disease (AD) is a common illness with an increasing incidence as populations get older. The disease is pathologically characterized by aggregates of amyloid β peptides (Aβ) and intercellular neurofibrillary tangles of tau proteins. Currently, no cure exists for AD, but many drug candidates are being developed, focusing both on the prevention or break-down of the protein aggregates and on treatment of the cognitive symptoms.

Pre-clinical research into AD primarily uses transgenic mouse models carrying human mutations in the APP and gamma secretase components PSEN1 and PSEN2 genes, leading to accelerated amyloid plaque formation. These models allow for monitoring of disease progression and screening intervention efficacy, although they are limited to the amyloid-related aspects of the Alzheimer’s pathogenesis.

Fluorescence imaging

Optical imaging methods are sensitive methods for in vivo imaging, based on the detection of photons emitted from the tissue of interest. For in vivo fluorescence imaging (FLI), fluorescent proteins or dyes are used that need external excitation for light emission, with wavelengths in the visible and near infrared parts of the spectrum. Optical imaging techniques are characterized by their high sensitivity and specificity towards the target of interest. However, the tissue penetration of visible light is limited, and scattering and absorption of the photons by tissue impair precise localization and quantification of the signal (Table 1).

In contrast to non-invasive whole body fluorescent imaging, techniques like multi-photon microscopy can be used to image intact tissues with sub-cellular resolutions [2]. Multi-photon techniques are based on exciting a fluorophore by simultaneous absorbance of two long wavelength photons rather than one short wavelength photon, which is the case...
with normal fluorescence. The use of long, near infrared, wavelengths and two photons simultaneously has several advantages compared to lower wavelength photons, such as better in-depth localization, deeper tissue penetration and lower photo-toxicity \[2\]. This technique requires the presence of an optical window, that is, an intracranial window when the brain is studied. The placement of this window requires a time-consuming operation procedure and makes the techniques more invasive than, for instance, magnetic resonance imaging (MRI). However, the advantage of the window is that the major source of inaccuracy in optical imaging, namely absorption and scattering by tissue, is greatly reduced, thus improving the quantification of the fluorescent intensity, although the measurements are restricted to the small part of the brain that lies directly under the window.

**FLI detection of amyloid depositions**

Imaging of \(\text{A}\beta\) aggregates in the brain is one of the most straightforward ways to follow AD disease progression. These dense core and diffuse \(\text{A}\beta\) deposits have been imaged using \(\text{A}\beta\)-specific antibodies coupled to a fluorescent label. Examples of such antibodies are alexa-fluor-750-conjugated BAM-10 \[3\] and fluorescein-labeled 10d5 \[2\]. Most of these ligands need to be applied locally, as they do not easily cross the blood–brain barrier (BBB). Figure 1 shows an example where multi-photon microscopy was used to image \(\text{A}\beta\) deposits in the brain, \(\text{A}\beta\) deposits on blood vessels and the microvasculature simultaneously.

Nowadays, mostly small amyloid-targeting fluorescent molecules are used, the most standard one being the Congo Red derivative methoxy-X04, which readily crosses the BBB and is administered via a straightforward intraperitoneal injection. Another well-known compound is Thioflavine-S \[3,4\], which not only binds to \(\text{A}\beta\) deposits, but also to neurofibrillary tangles.

These techniques have been used to study the beneficial effects of natural compounds like curcumin, an Indian spice with a broad spectrum of anti-oxidant, anti-inflammatory and anti-fibrilogenic activities. *In vivo* multiphoton microscopy showed that curcumin crossed the blood–brain barrier, and after systemic treatment of mice for seven days, curcumin cleared and reduced existing plaques. Curcumin labels amyloid pathology *in vivo*, disrupts existing plaques, and

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<tr>
<th>Table 1. Comparison of FLI and MRI</th>
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<tbody>
<tr>
<td><strong>Fluorescence imaging (FLI)</strong></td>
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<td>Whole body imaging</td>
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Figure 1. Multicolor fluorescence from the cortex of a PDAPP mouse. This composite image represents a three-color projection of a three-dimensional volume of the cortex of a living transgenic mouse. Thioflavine-S, displayed in red, labels dense-core amyloid deposits on blood vessels (e.g. traversing horizontally in the middle of the image) and plaques (yellow deposit, just above center). Fluorescein-labeled 10d5 antibody, displayed in green, labels dense-core and diffuse amyloid deposits in the brain. Texas red dextran (70 kDa), displayed in blue, was injected into a lateral tail vein to allow fluorescence angiography. This dye does not cross the blood–brain barrier and fills all blood vessels. All three dyes were excited simultaneously at 750 nm, and fluorescence emission was captured with a three-channel external detector system. The projected volume is about 150 mm deep from the surface of the cortex. The scale bar is 100 mm. (Reprinted with permission Fig. 2 from Bacskai et al. [2]).
partially restores distorted neurites in an APPswe/PS1dE9 Alzheimer’s disease mouse model [5].

Fluorescent probes like AOI987 and the curcumin-derivative CRANAD-2 are more recent additions to the arsenal of Aβ-targeting probes [6,7]. The fluorescence intensity, excitation/emission wavelengths and fluorescent lifetime of these probes change upon binding to Aβ [6,8]. These changes make it possible to selectively image the Aβ-bound probe, minimizing a-specific background signal due to the unbound probe present in the tissue. These properties are particularly useful for non-invasive whole-body FLI studies, where the resolution is not sufficient to detect the actual plaques.

Lastly, the recent development of transgenic mice that express luciferase under control of the Gfap promoter as well as mutant human amyloid precursor protein (Tg APP23:Gfap-luc) allows monitoring of Aβ accumulation without the need for an external fluorophore or light source (Fig. 2) [9].

**FLI detection of structural alterations**

Apart from direct detection of amyloid aggregates, optical imaging is a suitable modality to study the interaction between plaques and healthy brain tissue. Using fluorescein-labeled tomato lectin, Bacsai et al. imaged microglial cells surrounding dense plaques, up to a depth of about...
50 μm from the surface of the brain of living Tg mice that overexpress human APP [2]. In another study, D’Amore et al. looked at the structural changes of neuronal cells in close proximity to plaques in PDAPP mice. Diffuse amyloid and dense core plaques were labeled in vivo with fluorescein-conjugated antibodies and Thioflavine-S, and a set of neurons and neurites was labeled using dextran-lysine conjugated Alexa Fluor 594. Multi-photon imaging revealed structural changes such as increased curvature and bulbous outgrowths in neuronal processes near Aβ deposits [10]. As an example of the use of these techniques in drug research, Spires-Jones et al. recently published a paper detailing the acute effect of passive immunotherapy on dendritic spines. Within one hour of applying a neutralizing anti-Aβ antibody, they showed a small but significant increase in dendritic spine formation in PDAPP mice brain far from plaques, demonstrating that removing toxic Aβ species rapidly increases structural plasticity [11].

Magnetic resonance imaging
In contrast to optical imaging, MRI is characterized by its high anatomical resolution and intrinsic contrast, combined with virtually unlimited tissue penetration. As such it is one of the most frequently used imaging modalities in pre-clinical, and clinical, in vivo characterization of AD. Analogous to FLI, MRI can be used to image Aβ depositions in the murine brain, but is in addition very well suited to monitor other AD-related morphological and functional alterations.

MRI detection of amyloid depositions
Amyloid beta plaques of over 100 μm in diameter can be directly detected by MRI as hypointense spots in high-resolution T2 or T2* weighted images [12]. Figure 3 shows how circular hypointense spots on MRI co-localize with ex vivo immunohistochemistry staining against Aβ depositions. It has been postulated that the MRI hypointensities are the direct result of iron deposition inside the dense core plaques. Iron is indeed found in many senile plaques, and increasing iron content in the brain, along with other metals, correlates with the increase in beta amyloid burden [13]. However, iron does not deposit uniformly in individual plaques, nor does it deposit in all plaques present in the murine brain. Furthermore, the deposition of metals in the individual plaques does not occur until the disease has progressed into late stage, and is not found in younger animals [13,14]. On top of that,
T2/T2* weighted hypointensities are not limited to AD and most amyloid plaques are smaller than 100 μm, which all may lead to misidentification of the observations [15]. Finally, as a consequence of the generally small amyloid plaque size, the long scan times that are often necessary to achieve acceptable resolution may make the procedure challenging for routine application in in vivo pre-clinical studies [16].

As a result, attempts have been made to facilitate a reliable in vivo detection of individual Aβ depositions by the use of specific MRI contrast agents. Such contrast agents can be gadolinium chelates or superparamagnetic iron oxide nanoparticles tagged to amyloid peptides or to anti-Aβ monoclonal antibody fragments [17–19] or even untargeted gadolinium [20]. Also FSB, a fluorinated amyloidophilic compound closely related to the optical imaging agent methoxyXO4, and detectable by 1H and 19F MRI has been reported to target plaques in 12- to 24-month-old APP Tg mice [21].

However, up to now all of the above mentioned Aβ-specific contrast agents rely on the co-injection of mannitol or DMSO to artificially open the blood–brain barrier (BBB), or employ intra-cranial injection of the agent, effectively reducing the non-invasive character of MRI and limiting longitudinal studies.

As an alternative to monitoring individual plaques, it is possible to quantify greater regions of interest. Teipel et al. described an automated, voxel-based analysis of T2 relaxation time changes in the entire brain of an APP/PS1 transgenic AD mouse model. While this approach does not allow visualization of individual plaques, it is applicable at lower field strengths and can be easily quantified, without the use of any contrast agents and is not limited to a priori selected areas [22].

Considering the current Aβ cascade hypothesis, monitoring altered Aβ deposition buildup over time may be the most direct and specific method for the effect of anti-AD drug intervention, and drug development as such. However, the strength of MRI is that it is also applicable for measurements of the consequences of amyloid aggregates on brain structure and function.

**Detection of brain atrophy**

In clinical studies and diagnosis of AD, the occurrence of cerebral atrophy is well characterized and volumetric assessments are used routinely on patients with mild cognitive impairment (MCI) and AD. By contrast, brain atrophy in AD mouse models is more difficult to detect and subject to several challenges, among which the fact that the murine brain is approximately a 3000-fold smaller than the human counterpart and the high contrast between gray and white matter in the human brain is absent in mice [23]. But in an APPswe/ind AD mouse model, Badea et al. demonstrated a proof-of-principle that, as in human MCI and AD patients, amyloid deposition is preceded by MRI detectable cortical volume loss [24]. With the development of new tauopathy murine models, in which brain atrophy seems more predominant, and continued development of scan and analysis techniques and protocols, volumetric MRI may become an efficient translational imaging tool in pre-clinical AD drug development [25].

**Detection of microvascular alterations**

Much effort has been put into imaging the functional consequences of amyloid deposits.

One such approach employs the increased presence of macrophages and microglia around vessels heavily affected with cerebral amyloid angiopathy (CAA), the vascular deposition of Aβ [26]. Untargeted iron oxide nanoparticles are being taken up by the macrophages through absorptive endocytosis and subsequently provide enhanced contrast, allowing non-invasive monitoring of the development of vascular pathology, correlated with Aβ build-up. Using this method, in combination with histological post mortem analysis, Beckmann et al. showed how prolonged passive Aβ immunotherapy treatment increased the incidence of vascular lesions colocalizing with iron-loaded macrophages in APP23 mice that develop CAA.

Also the increase of BBB permeability can be detected on MRI. Contrast enhanced T1-weighted images shows leakage of the contrast agent gadolinium-DTPA into tissue surrounding CAA-affected vessels [27]. By increasing the phagocytosis of amyloid by macrophages, Lifshitz et al. have been able to reduce the leakage in a TGF-β1 transgenic mouse model back to WT control levels and thereby demonstrated the value of this method in drug development.

A clinically well-known feature of CAA is the occurrence of microhemorrhages. These microbleeds are also present in some transgenic AD models, and can be detected using T2*-weighted scans without the use of contrast agents [28]. Susceptibility-weighted MRI is applied in clinics to detect microhemorrhages in AD patients and as such, this technique, applied in murine models, is supplied with a potentially well-established translational platform. Microhemorrhage susceptibility is a risk factor for vasogenic edema, correlated with Aβ immunotherapy in (pre-)clinical trials and the ability to monitor their incidence may be crucial for successful therapy development [28].

On a microvascular level, MRI measurements of cerebral perfusion monitor the delivery of arterial blood to the capillary bed. As such it has been found that perfusion and cerebral blood volume are decreased in transgenic models of AD [29]. Mechanistically this could lead to a state of hypoperfusion, and thereby exacerbate the cognitive symptoms. Cerebral perfusion measurements have, for example, been applied to determine the effect of dietary lipids on brain circulation in APP/PS1 Tg-mice, showing how increased intake of dietary...
docosahexaemomic acid, or DHA, is likely to increase relative cerebral blood volume but not blood flow [30]. On a somewhat larger scale, blood flow analysis or magnetic resonance angiography (MRA) can be employed to follow the alterations of bigger blood vessels in transgenic mouse models. The inflow of water protons inside the vessels is used to generate high contrast with respect to residual signal from saturated protons in surrounding stationary tissue, a technique known as time of flight (TOF-) angiography. A drawback of TOF-angiography is that the smaller veins and arterioles remain practically undetected due to low flow rates. In contrast-enhanced (CE-) angiograms, the use of a paramagnetic contrast agent in the bloodstream allows higher contrast and more detail, up to the level of the veins, and may allow visualization of blood vessels which are not detected by TOF-MRA [31]. Using MRA, Thal et al.

Figure 4. MRA images of a 26-month-old, male wild-type mouse without flow disturbances (a: anterior–posterior projection; b and d: oblique projection) and a 26-month-old, male APP23 mouse with evident alterations of cerebral blood flow (c and e: oblique projection). (a, b and d) All vessels in the wild-type mouse exhibited a conspicuous blood flow signal (arrows in a, b and d). There was no evidence for stenosis or occlusion of the vessels. The posterior cerebral artery branches into the superior cerebellar artery (SCA), the thalamoperforating artery (TPA) and the main part of the posterior cerebral artery (ACP) best seen in an oblique projection (b and d). In the APP23 mouse the TPA and the main part of the ACP were not detected distal to the trifurcation (large arrow in c and e) indicating disturbances in cerebral blood flow. The small arrows in (e) point to the area where TPA and ACP were seen in the non-transgenic mouse (Reprinted with permission Fig. 5 from Thal et al. [32]).
demonstrated how wild-type mice did not show the blood flow disturbances that were found in their APP23 transgenic model, as shown in Fig. 4 [32].

Detection of functional alterations
In addition to monitoring buildup of Aβ and alterations in the cerebral vasculature, it is also possible to use MRI to look at the functional consequences of progressive AD in rodent models and the effects of experimental treatment.

One such approach recognizes the fact that in triple transgenic mice, axonal transport impairment has been linked to the pathogenic processes of the disease. Manganese-enhanced MRI (MEMRI) utilizes the fact that the manganese ion (Mn²⁺) is a paramagnetic calcium analog, which is competitively taken up into neurons via voltage-gated calcium channels, packaged into vesicles and transported down the axon. Therefore, manganese chloride (MnCl₂) can be used as a contrast agent to map the overall axonal transport integrity [33]. For example, it has been used to demonstrate the positive effect of the Aβ42 selective lowering agent R-flurbiprofen on axonal transport in the Tg2576 (SweAPP695[K670N/M671L]) mouse model. In this study, the authors show how reductions in both Aβ42 levels and tau hyperphosphorylation as a result of chronic R-flurbiprofen treatment correlate with improved transport as assessed by MEMRI [34].

Metabolic changes can be assessed using ¹H MR Spectroscopy (MRS). Using MRS, metabolites can act as functional markers, for example, lactate for impaired metabolism and cerebral blood flow, N-acetylaspartate for neuronal number, integrity and health and thus for neurodegeneration, myo-inositol for glial cells and cerebral osmolarity, although absolute quantification is challenging [35]. Using MRS it has been determined that in an inducible Tau transgenic model signs of gliosis, that is, increased myo-inositol, were indeed increased compared to wild-type [25].

Finally, a widely used approach in clinical cases of neurodegenerative disorders is functional MRI or fMRI. This very interesting tool still has many technical challenges to overcome for useful pre-clinical application, including the limited knowledge on the effects of anesthetic agents and the lack of relevant challenging paradigms. To partly circumvent these issues, it is possible to trigger brain activation by electrically or pharmacologically stimulating brain regions of interest, while simultaneously monitoring brain activation, for example, by using MEMRI, blood-oxygen level-dependent (BOLD) fMRI or CBV-based fMRI [23,36]. For instance, by applying cerebral blood volume-based fMRI Princz-Kranz et al. determined the diminished response of cerebral blood vessels to the vasodilatory compound acetazolamide in aged arcAβ Tg-mice, a model that develops both parenchymal and vascular depositions of Aβ. The altered fMRI response with respect to wild-type animals may serve as a diagnostic biomarker for cerebral Aβ deposition [37].

Conclusion
Both FLI and MRI have proven to be effective in the in vivo detection of Aβ depositions and related alterations, but both have issues which should be addressed. FLI has excellent contrast agents, some even able to cross the blood–brain barrier independently, but lacks the penetration depth found in MRI. Attenuation of light by tissue greatly interferes with its application. Emerging transgenic bioluminescent models may improve at least some of these problems.

Alternatively, a cranial window in the mouse model certainly alleviates this problem; however, the procedure is invasive, requires a time-consuming operation procedure and limits the view to the underlying cranial region and may therefore not yet be easily applied in routine drug development research.

MRI, by contrast, requires expensive equipment and specialized personnel, even although it may still score better on these aspects than for instance PET imaging. Its intrinsic contrast can be applied to the detection of larger plaques; however, without the aid of contrast agents, confident detection of amyloid plaques in the murine brain is very challenging. Region-of-interest analyses to detect T2 changes associated with iron accumulation around plaques will probably be more time-efficient and allow for higher throughput. Contrast agents for MRI are being developed, but lack the sensitivity and the efficient BBB passage found in FLI contrast agents. Co-injection with BBB modulating agents such as mannitol is sufficient to bypass this problem, but ideally this would not be necessary.

MRI combines high resolution with good soft tissue contrast and is very well suited to detect both functional and anatomical alterations associated with AD. Moreover, MRI can examine the structure of the whole brain in any plane and generate three-dimensional images suitable for detailed volumetric quantification. Alterations in metabolites, cerebral blood flow and brain activation can be measured using MRI. These techniques are currently applied in clinical settings as well, which makes eventual translation to a clinical setting easier. The development of cost-efficient MRI systems, such as the low-field Bruker Icon™ (1T) and bench-top Vision MRI, and the introduction of improved detection techniques such as the highly sensitive but rather costly cryogenic radio-frequency probes [38], will open up the MRI field to more researchers and better detection of amyloid depositions.

As shown here with examples of recent publications, currently both imaging modalities, FLI and MRI, yield studies validating the detection of Aβ depositions or related alterations, sometimes using a validated pre-clinical therapy method such as antibody treatment to strengthen the evidence. These studies have yielded novel contrast agents or imaging
procedures, but up to now not a single novel therapeutic has been introduced using these imaging modalities. This is in part due to the common acceptance that the gold standard in defining AD, and thus effect of treatment, is neuropathology [39]. Hence all statements made above were backed up by ex vivo immunohistochemistry, and the studies are actually proof-of-concepts of the detection method. A second problem may be that currently no common standard has been proposed for pre-clinical diagnosis of AD phenotype based on MRI or FLI. A next step towards universal pre-clinical diagnosis and monitoring of AD would be to standardize protocols and to introduce between-site validation of the imaging methods. On such a basis, drug development in search for in vivo diagnosis and cure of AD could be more straightforward, reproducible and, importantly, comparable.

Conflict of interest
The authors have no conflict of interest to disclose.

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