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Spectral unmixing imaging of wavelength responsive fluorescent probes: an application for the real-time report of amyloid beta species in Alzheimer's disease

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Abstract

We investigated a method for the real-time assessment of a target concentration *in vivo* using a combination of a spectral unmixing technique and a fluorescent probe (CRANAD-3), which is specific for amyloid-beta ($A\beta$) species, the biomarkers for Alzheimer's disease (AD), and has a significant emission wavelength shift upon binding to $A\beta$ species. With our approach we were able to differentiate bound probe from unbound probe in a phantom, *ex vivo* in brain slices and whole brain, and *in vivo* in a transgenic mouse model of AD. In the phantom study, the fluorescence intensity of the unmixed bound signal was tightly correlated with the concentration of $A\beta$. Unmixed images of AD mouse brain slices revealed that bound CRANAD-3 specifically distributed across the cortical regions and hippocampus. *Ex vivo* unmixing imaging of whole AD brain clearly showed that the bound probe was primarily located in the cortex while unbound probe was mainly present in blood vessels, distributed across the brain and in the heart. Remarkably, the *in vivo* unmixed signals of bound CRANAD-3 reached a plateau with increasing dosage, demonstrating that these signals correspond to $A\beta$ content, not probe injected dose. Time-course imaging results indicated that the bound signals were substantially steady at 10, 20, and 30 minutes post-injection, providing additional evidence that signals processed by the spectral unmixing technique could be used as a real time reporter of $A\beta$ species loading *in vivo*.

Introduction

Differentiation of bound and unbound fluorescence probes *in vivo* is an immense challenge in imaging research. Normally, quantification of the signal of a bound probe is conducted after washing out any unbound, free probe. However, this washout step may result in the unintentional clearance of a certain fraction of bound probe, resulting in underestimation of the true concentration of the target. To produce a real-time report of target concentration *in vivo*, we propose a method that combines a spectral unmixing technique^{1–5} with application of a wavelength responsive fluorescent probe^{6–9}. The wavelength responsive probe's fluorescent spectrum is distinct in its bound and unbound state, thereby enabling the potential use of spectral unmixing techniques to resolve raw image signal into individual spectra corresponding to background, bound probe and free probe signals.

Spectral unmixing, a dissecting algorithm, is able to partition crude fluorescence signals originated from multiple fluorophores into individual contributors. Although spectral unmixing is widely used in fluorescence microscopy for cell imaging^{4,5,10}, its *in vivo* applications are technically challenging. Tissue absorption and scattering can have a significant impact on the measured spectrum of a fluorophore, making the *in vivo* spectrum considerably different from that *in vitro*. For *in vitro* microscopy imaging, signals are from fluorophores diluted in a thin transparent solution or collected directly from a very thin

focus plane. Therefore microscopic imaging could be considered as 2D imaging. For in-vivo imaging, however, signals are the diffuse radiance from the fluorophore source embedded in scattering animal tissue, which makes a significant contribution to a cloud of surrounding pixels instead of only the pixel geometrically associated with the fluorophore. Therefore, in vivo imaging could be considered as a complex 3D problem. Recently, Xu et al. showed that a multivariate curve resolution (MCR) method could be used as a spectral unmixing algorithm for *in vivo* optical imaging that does not rely on precharacterized spectral libraries. More importantly, their MCR algorithm could solve the complex 3D approximation by using measured 2D spectral data. They have successfully demonstrated the technique by unmixing autofluorescence and two different fluorescent probes in nude mice ¹.

We have previously reported that several curcumin-based near infrared fluorescence probes were “turn on” probes for amyloid beta (A β) species ^{7,11,12}, the most important biomarkers for diagnosis of Alzheimer’s disease (AD) ^{13,14}. Probe binding to A β species led to significant changes in several fluorescent properties, including fluorescence intensity, emission peak, lifetime and quantum yield. In this study, we utilized the emission wavelength change of curcumin probe and spectral unmixing techniques to distinguish between the spectra of the bound and unbound probe upon binding to the target. We believe that this method provides a way to perform a real-time assessment of A β species *in vivo*.

Methods

Reagents used for synthesis were purchased from Aldrich and used without further purification. Column chromatography was performed on silica gel (SiliCycle Inc., 60 Å, 40–63 mm) slurry packed into glass columns. Synthetic amyloid- β peptide (1–40/42) was purchased from rPeptide (Bogart, GA, 30622). ¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz respectively, and reported in ppm downfield from tetramethylsilane. Fluorescence studies were carried out using a F-4500 Fluorescence Spectrophotometer (Hitachi). Transgenic APP-PS1 mice and age-matched wild-type mice were purchased from Jackson Laboratory. All experimental procedures were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. *In vivo* NIR imaging was performed using the IVIS®Spectrum animal imaging system (Caliper LifeSciences, Hopkinton, MA), and spectral unmixing was conducted using LivingImage® 3.2 software.

Synthesis of CRANAD-3

The synthesis of the immediate material (2,2-difluoro-1,3-dioxaboryl-pentadione) was performed using a modified procedure ⁷. The immediate compound (0.15g, 0.1mmol) was dissolved in acetonitrile (3.0 ml), and acetic acid (0.2ml), tetrahydroisoquinoline (0.04mL, 0.3mmol), and 6-*N,N'*-diethyl-3- pyridylaldehyde (0.36g, 2.0mmol) were added. The resulting solution was stirred at 60°C overnight. A black residue was obtained after removing the solvent, and was subjected to flash column chromatography with methylene chloride to produce a black powder (63.0mg, yield: 15.0%). ¹H and ¹³C NMR spectra were recorded at 500 MHz and 125 MHz respectively, and reported in ppm downfield from tetramethylsilane. ¹H NMR (CDCl₃) δ (ppm) 1.24 (t, 12H, J = 7.2 Hz), 3.58 (q, 8H, J = 7.2, 14.0 Hz), 5.89 (s, 1H), 6.42 (d, 2H, J = 16.0 Hz), 6.51 (d, 2H, J = 9.0 Hz), 7.66 (dd, 2H, J = 2.4, 9.0 Hz), 7.92 (d, 2H, J = 16.0 Hz), 8.34 (d, 2H, J = 5.0 Hz); ¹³C NMR (CDCl₃) δ (ppm) 18.8, 42.7, 101.6, 106.3, 115.9, 118.6, 136.0, 144.2, 153.4, 159.8, 178.2.

Phantom imaging

A 1.0ml PBS solution of A β 42 (250 nM) was added to each well of a 12-well plate, followed by the addition of a DMSO solution (10 μ L) of CRANAD-3. The final

concentrations of CRANAD-3 added were 50, 100, 250, 500, 750, and 1000 nM, with samples prepared in quadruplicate. The resulting plate was subjected to imaging using IVIS®Spectrum by setting Ex = 530nm, Em = 580nm to 840nm with 14 filters, subject height = 0.2cm, and FOV = C. Spectral unmixing was performed with LivingImage® 3.2 software by setting Component = 3 (representing 3 fluorophores that include autofluorescence, bound CRANAD-3 and free CRANAD-3), Photo Mask (this tool is for selecting the area of interesting), and Auto constraints (this tool is for constraining parameters to allow user zeroing low band pass and making non-negative contribution from fluorophores. In our experiments, we used the auto default model from the imaging system).

Tissue staining and imaging

Wild type (20-month old) or APP/PS1 (14-month old) mouse brain tissue was cut into 25-micron slices, fixed in 4 % formalin for 5 min and washed twice with PBS buffer. The tissue was incubated with 0.01% CRANAD-3 (in 50% ethanol) and washed. The tissue was subjected to imaging using the same parameter settings as were used for the phantom imaging.

Ex vivo brain imaging

A 24-month APP/PS1 mouse was intravenously injected with 1.5mg/kg CRANAD-3 (freshly prepared with 15% DMSO, 15% cremophor and 70% PBS buffer). After 1 hour, the mouse was sacrificed and the brain, heart, and spinal cord were collected. The three tissue/organs were subjected to imaging with Ex = 530nm, Em = 580 – 840nm, small binning, f = 2, FOV = B, and exposure time = 0.5s. Spectral unmixing was performed using LivingImage® 3.2 software by setting component = 3, Photo Mask, and Auto constraints.

In vivo imaging

Imaging was performed using an IVIS®Spectrum optical system (Hopkinton, MA). For dosage testing, APP-PS1 mice (n = 3) were shaved, and intravenously injected with 0.25, 0.5, 0.75, 1.0mg/kg of freshly prepared CRANAD-3 in 15% DMSO, 15% cremophor and 70% PBS. Fluorescence signals from the brain were recorded at 30 minutes after the injection of the probe. To monitor the progression of AD pathology (11-month and 19-month old APP-PS1 mice, n = 5), image sequences were recorded at 10, 20, 30, 40, and 50 minutes post-injection. Imaging parameters were set at Ex = 570nm, Em = 620 – 840nm, medium binning, f = 4, FOV = C, and exposure time = 1s. Spectral unmixing was performed with LivingImage® 3.2 software by setting Component = 3, Photo Mask, and Auto constraints. To evaluate imaging results, an ROI was drawn around the brain region. For the bound signal, the ROI was drawn on the unmixed image corresponding to the bound spectrum, and total efficiency, which is fluorescence emission image normalized to the incident excitation intensity (radiance of the subject/illumination intensity), was used for quantification.

Results

1. Phantom unmixing imaging with synthetic A β 42 monomers

CRANAD-3 is a curcumin derivative that displays properties changes after binding to A β species. In particular, there is a 60 nm blue shift of the emission peak upon binding to synthetic A β 42 (Fig. 1A). To demonstrate the proof-of-principle that CRANAD-3 could be used together with the spectral unmixing technique, we incubated a fixed concentration of synthetic A β 42 monomers (250nM for all the wells) with varied concentrations of CRANAD-3 (50 – 1000 nM, see Methods for details). The plate was imaged using an excitation filter at 535 nm, and emission signal in the range of 580–840 nm (raw images are

shown in SI Fig. 1). We performed spectral unmixing for this plate to resolve bound CRANAD-3 and free CRANAD-3. The unmixed distribution images are shown in Fig. 1B. As seen in Fig. 1C, the two spectra were clearly resolved after unmixing. Furthermore, the spectra of bound and free CRANAD-3 closely resembled the spectra recorded using a fluorescence spectrophotometer (Fig. 1A), with the spectrum peak for the bound probe around 640nm and the spectrum peak for the free probe around 700nm. The intensity of unmixed bound signal increased with increasing concentration of CRANAD-3, which ranged from 50nM to 250nM. However, the bound signal did not significantly increase with concentrations of CRANAD-3 ranging from 250nM to 1000nM. In contrast, the unmixed signal for free probe increased with all concentrations of CRANAD-3.

Quantifications of the efficiencies of the signals, in which: Efficiency (%) = emission light (photons/second)/excitation light (photons/second), were conducted at different probe concentrations (Fig. 1D). Consistent with the unmixed images, the quantified bound signal did not significantly change with increases in probe concentration once A β reached a fully-bound, saturated state. However, the free probe signal increased with all probe concentrations, suggesting the unmixed bound signal was tightly correlated with the concentration of A β but not with the concentration of the probe added.

2. Imaging of CRANAD-3-stained brain slice from a wild type mouse

To test whether the spectral unmixing technique was capable of differentiating the binding status of probe in brain tissue, we stained a brain slice from a 20-month-old wild type mouse with no A β pathology with CRANAD-3 and then imaged the slice using similar imaging parameters as the phantom imaging. In raw images of the slice, we found that the signal from the corpus callosum, the largest white matter structure of the brain, was slightly higher than in other regions (Fig. 2A), suggesting that CRANAD-3 had some interaction with white matter. To test whether the unmixed spectrum for white matter was different from the spectrum of CRANAD-3 bound to A β determined from phantom studies, we unmixed the raw images into three components, representing autofluorescence, white matter binding, and free probe spectra. After unmixing, we found that the peak for white matter binding was around 600 nm, a 40 nm difference from that of the A β bound spectrum from phantom studies (Fig. 2B and Fig. 1C). This was not surprising given that fluorescence probes are known to display a higher intensity and blue shift upon interaction with hydrophobic environments¹⁵ like the environment of white matter tissue. We found that the peak for the free probe (680nm) was 20 nm shorter than that of the phantom (700nm), a finding likely due to the differences in environment between phantom solution and brain slices (Fig. 2B). The distribution map likewise showed that the CRANAD-3 bound to white matter was mostly present in the corpus callosum area (Fig. 2C #2 and #4), while the free probe was distributed randomly across the tissue (Fig. 2C #3). Our results suggest that spectral unmixing coupled with wavelength responsive probe CRANAD-3 allows us to spectrally and spatially differentiate white matter binding from free probe while providing evidence that binding to non- A β structures can be differentiated from A β binding by a spectral peak shift.

3. Ex vivo imaging of a brain slice from APP/PS1 mouse

APP/PS1 mice, a widely used transgenic strain for AD research, express chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 protein (PS1-dE9). The “humanized” Mo/HuAPP695swe transgene allows the mice to secrete a human A β peptide, leading to a gradual increase A β loading during the aging process^{16,17}. In our next experiment we used brain slices of a 14-month APP/PS1 transgenic mouse obtained after intravenous injection of CRANAD-3. Interestingly, the raw images of an ex vivo slice showed weak white matter binding (Fig. 3A), indicating that at

the time of imaging (2 hours post-injection) most CRANAD-3 had washed out of the white matter. As expected, we obtained well-resolved spectra and images after performing the unmixing procedure (Fig. 3B, C) corresponding to spectral signals of autofluorescence, A β bound probe and free probe. From Fig. 3B, it can be seen that the bound probe primarily distributed in cortex area and hippocampus, and this distribution pattern was similar to the A β plaques deposit pattern observed from fluorescent microscopic images (SI Fig.3). The spectrum peak of bound probe was 640 nm, the same as the peak obtained in phantom imaging studies (Fig. 1C). Similarly, the spectrum of free probe was very similar to that of *in vitro* CRANAD-3 stained wild type brain slice (Fig. 2B).

4. Ex vivo imaging of a whole brain from APP/PS1 mouse

After we demonstrated that the spectral unmixing technique was feasible for brain tissue slices using our CRANAD-3 probe, we tested this technique on the whole brain *ex vivo*. To this end, we obtained the non-perfused whole brain from a 24-month old APP/PS1 mouse 1 hour after intravenous injection of CRANAD-3. The brain, heart and a portion of spinal cord were imaged as described previously. After unmixing of the raw images, well-resolved spectra were generated for autofluorescence, bound and unbound CRANAD-3 (Fig. 4A). The spectra were similar to those obtained after AD tissue imaging. Remarkably, the unmixed images clearly showed that the bound probe was primarily located in the cortex (Fig. 4B #2), while the unbound probe was present in the blood vessels, across the whole brain and in the heart (Fig. 4B #3). The composite image, the merged/summed image of the images of the unmixed components, indicated that the probe in the blood vessel and the heart primarily existed in its unbound form (red) (Fig. 4B #4), whereas the probe in the cortical regions was bound (blue). Additionally, we noticed that the probe in the spinal cord was mainly present as a bound form (blue) while the blood in the narrow tissue area surrounding the spinal cord contained an unbound probe (red). These unmixed images reflected the fact that A β species were primarily present in the cortex and cerebrospinal fluid (CSF) and that their concentration in the blood was considerably lower. In addition, we did not observe the spectral peak for white matter binding. Several things could account for this, including the deeper location of the white matter, washing out of the probe from the white matter, or the embedding of the white matter binding signal into the spectrum of autofluorescence.

5. Differentiating wild-type and transgenic AD mice by spectral unmixing in vivo

The intrinsic difference between wild-type and transgenic AD mice is the absence of A β species in the wild-type^{16,17}. We reasoned that free probe would be a dominant component in wild-type mice, while probe bound to A β species would predominate in APP/PS1 mice. To test this hypothesis, we intravenously injected both wild-type and APP/PS1 mice with CRANAD-3, and imaged them with excitation at 570 nm and emission ranging from 620–840 nm. We selected the 570 nm filter excitation for *in vivo* imaging because of its better tissue penetration than 535 nm, the excitation filter for *ex vivo* imaging. We unmixed the raw images using autofluorescence and unbound free probe as two expected major components in wild-type mouse. There was a peak in the unmixed spectra around 670 nm (Fig. 5B) corresponding to the free probe existing in an unbound form. The unmixed spectrum for APP/PS1 mouse was very similar to the spectrum of A β bound probe, suggesting that the major component existed in the bound form (Fig. 5A). This result indicates that we could use the spectral unmixing technique to differentiate wild-type from transgenic AD mice.

6. Real time report of A β loading and monitoring the progression of amyloidosis pathology in APP/PS1 mice

In the phantom imaging experiment, we showed that the unmixed bound probe signal was tightly correlated with the content of A β , suggesting that this unmixing technique could be used to report the concentration of a target in real time. To test the feasibility of this approach *in vivo*, we injected 16-month old APP/PS1 mice with different dosages of CRANAD-3 (0.25–1.0 mg/kg), and imaged 30 min post-injection (a representative raw image sequence is shown in SI Fig. 2). We then unmixed the raw images into the spectra of three components corresponding to autofluorescence, A β bound probe and free probe. As expected, distinct unmixed spectra were obtained for all three components, with the peak for bound probe at 640nm and the peak for free probe at 680nm (Fig. 6B). These peaks corresponded well with the results of *ex vivo* whole brain imaging. Semi-quantitative analysis of the unmixed images was conducted by selecting the whole brain area as a region of interest (ROI) and monitoring total efficiency at 30 min post injection as the readout. Analysis of the raw images showed that the raw signals at 640 nm increased with the increasing dosages of the probe (Fig. 6C, black line). The unmixed signal for the bound probe increased with the increasing probe dosage from 0.25mg/kg to 0.5mg/kg. However, at dosages of 0.5, 0.75 and 1.0mg/kg, the unmixed signals were not substantially different (Fig. 6C, red line). This result indicated that 1) the target A β was saturated around the dosage of 0.5mg/kg, and 2) the unmixed signals were closely correlated to the loading of A β and independent of injection dosages once the target was saturated. This result was consistent with the results of phantom imaging, in which the unmixed A β bound signal was tightly associated with the concentration of A β species.

Next, we attempted to image differential A β loading in APP/PS1 mice of different age groups (11-month and 19-month old) after injection of CRANAD-3 using our approach. Analysis of the unmixed images displayed significant differences in the bound signal between the two groups. The signal from the 19-month old mice was 1.3-fold higher than that of the 11-month old mice at 10, 20, and 30 minutes (Fig. 6D). As seen in Fig. 6D, the readings at 10, 20, and 30 minutes were very consistent for both groups, indicating that the target was steadily saturated, while a partial wash out of the bound probe could be observed around 40 min. We believe that this saturated state reflects the A β loading *in vivo*. Our results further indicate that it is feasible to use the spectral unmixing technique to monitor the increasing A β loading associated with age, thus providing the ability to monitor the disease progression in AD mice.

Discussion

For intensity-based NIR imaging in the absence of a “smart” probe, *in vivo* differentiation between wild type and transgenic mice always depends on the retention the probe, which required significant time¹⁸. However, a probe with a “smart” property (such as intensity increase upon interaction with the target) could be used to differentiate wild-type and transgenic mice at the very early time points after injection⁷. Yet, the signals from these intensity-based images cannot reflect the real time concentration of the target, since it represents the sum signal from both bound and free probe. For wavelength-based imaging, the spectral unmixing technique could be used to resolve multiple components. When this technique was coupled with a probe possessing a significant emission wavelength shift upon binding to a target, it allowed for differentiation between bound and unbound probe. Therefore, the signal from the bound probe in this case reflects the real time concentration of the target if the probe saturates the target. In this report, we demonstrated that this technique is not only able to differentiate transgenic and wild type mice by comparing their unmixed spectra, but also able to report the A β content in real time once the A β was saturated.

Although spectral unmixing techniques have been widely used in microscopic imaging for more than ten years, its *in vivo* application in whole organs or the body has just been reported recently ¹. In principle, this technique could allow us to distinguish infinite components that have different spectra; however, in practice, its capacity is limited by the filter bandwidth and simulation method. In this report, we used the emission filter bandwidth of 20nm, such that any spectral differences less than 20nm could not be well resolved. For microscopic spectral unmixing, linear unmixing is used, a procedure that is not suitable for *in vivo* whole body imaging of mice. Recently, Xu et al. reported that MCR (multivariate curve resolution) could be used for *in vivo* spectral unmixing ¹. In this report, we also demonstrated that MCR in combination with a wavelength responsive fluorescent probe could be used for *in vivo* spectral unmixing imaging.

In vivo spectral unmixing techniques are still in an early stage of development. Significant improvements for both imaging hardware and processing software are highly desirable. We believe that better spectral resolution, achieved by using continuous emission collection and improved software, will provide more robust signal separation and more reliable quantification analysis.

In summary, we demonstrated that a spectral unmixing technique in conjunction with wavelength responsive fluorescent probe CRANAD-3 enabled us to differentiate autofluorescence, A β bound probe, and free probe *in vitro* and *in vivo* in a mouse model of Alzheimer's disease. The unmixed spectra of A β bound probe and free probe were in a good accordance in various samples, including phantom solution, *in vitro* stained slice, *ex vivo* slice, *ex vivo* whole brain, and *in vivo* mice. Furthermore, this method was also able to differentiate between wild-type mice and transgenic AD mice, and the tight correlation between bound signal and A β content provided a basis for real time reporting of A β load *in vivo*. Moreover, we showed that with this unmixing technique, we were able to detect the A β content changes during the progression of the disease. Finally, we believe that this technique could be extended to other wavelength responsive probes that display wavelength shift characteristics upon interaction with corresponding substrates ^{19–21}.

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Fig. 1.

(A) Normalized fluorescence spectra of CRANAD-3 alone (black line) with Aβ species (red line) in PBS solution, and chemical structure of CRANAD-3; (B) Unmixed distribution images of the bound probe (top) and the free probe (bottom); (C) Unmixed spectra of the bound probe (blue) and the free probe (red); (D) Unmixed signals (efficiency) of bound CRANAD-3 (red) and free probe (black) with respect to the concentration (nM) of CRANAD-3 added.

Fig. 2.

(A) Raw image ($E_m=600\text{nm}$) of wild-type mouse brain tissue; (B) Unmixed spectra of autofluorescence (green), white matter bound probe (blue), and free probe (red); (C) Unmixed distribution images of autofluorescence (#1), white matter bound probe (#2), free probe (#3), and composite (#4).

Fig. 3. (A) Raw image ($E_m=640\text{nm}$) of ex vivo APP/PS1 mouse brain tissue; (B) Unmixed distribution image of bound probe; (C) Unmixed spectra of autofluorescence (green), bound probe (blue), and free probe (red).

Fig. 4.
 (A) Unmixed spectra of autofluorescence (green), bound probe (blue), and free probe (red);
 (B) Unmixed distribution images of autofluorescence (#1), bound probe (#2), free probe (#3), and composite (#4).

Fig. 5.

(A) Unmixed spectra of autofluorescence (green) and bound probe (blue) in APP/PS1 mice;
(B) Unmixed spectra of autofluorescence (green) and free probe (blue) in wild-type mice.

Fig. 6. (A) Unmixed distribution images of autofluorescence (#1), bound probe (#2), free probe (#3), and composite (#4); (B) Unmixed spectra of autofluorescence (green), bound probe (blue), and free probe (red); (C) Fluorescence signal of raw images (black line) and unmixed bound probe (red line) from mice injected with different dosages of the probe; (D) Fluorescence signals of unmixed bound probe from 11-month and 19-month old APP/PS1 at different time points after i.v. injection of CRANAD-3.