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Development of new fusion proteins for visualizing amyloid- β oligomers *in vivo*

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The intracellular accumulation of amyloid- β (A β) oligomers critically contributes to disease progression in Alzheimer's disease (AD) and can be the potential target of AD therapy. Direct observation of molecular dynamics of A β oligomers *in vivo* is key for drug discovery research, however, it has been challenging because A β aggregation inhibits the fluorescence from fusion proteins. Here, we developed A β_{1-42} -GFP fusion proteins that are oligomerized and visualize their dynamics inside cells even when aggregated. We examined the aggregation states of A β -GFP fusion proteins using several methods and confirmed that they did not assemble into fibrils, but instead formed oligomers *in vitro* and in live cells. By arranging the length of the linker between A β and GFP, we generated two fusion proteins with "a long-linker" and "a short-linker", and revealed that the aggregation property of fusion proteins can be evaluated by measuring fluorescence intensities using rat primary culture neurons transfected with A β -GFP plasmids and A β -GFP transgenic *C. elegans*. We found that A β -GFP fusion proteins induced cell death in COS7 cells. These results suggested that novel A β -GFP fusion proteins could be utilized for studying the physiological functions of A β oligomers in living cells and animals, and for drug screening by analyzing A β toxicity.

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the progressive loss of cognitive functions. A typical neuropathological feature of AD is the deposition of senile plaques that are composed of the fibrillar amyloid β (A β) protein^{1,2}. Although extracellular A β deposition is well documented, emerging evidence indicates that A β also accumulates intraneuronally and might be critically involved in the progression of cognitive decline³. For example, intraneuronal accumulations of A β reduce the expression of synaptic proteins⁴, contribute to tau phosphorylation⁵, and mitochondrial dysfunction^{6,7}. Therefore the physiological functions of intraneuronal A β in non-fibrillar or water soluble forms have attracted increasing attention, and numerous reports have provided extensive evidence indicating that low molecular weight A β oligomers may act as the key molecule of the synaptic disorder⁸⁻¹⁵. The amyloid precursor protein (APP) E693 Δ mutant causes AD in humans. Expression of this mutant in mice resulted in age dependent accumulation of intraneuronal A β oligomers without formation of extracellular amyloid deposits, and induced synaptic and neuronal losses¹⁶. However, despite insights provided by biochemical, genetic, and animal model studies, effective therapeutic drugs that treat the symptoms of AD have not been developed. Direct observation of the process of accumulation and disaggregation of intracellular A β oligomers *in vivo* is critical for evaluating the efficiency of candidate therapeutic molecules and investigating the function of A β .

However, a major technical challenge is that it has been difficult to visualize A β in living cells when fused to the fluorescent proteins, such as GFP. Formation of the chromophore of fluorescent proteins depends on correct folding of the protein, and insoluble aggregation of the fused protein tends to cause loss of fluorescence¹⁷. Therefore, C-terminal fusion proteins containing wild type A β_{1-42} joined to GFP normally does not fluoresce, probably because A β_{1-42} aggregation results in GFP misfolding. Mutagenesis in the hydrophobic region of A β_{1-42} ,

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which contains the determinants of A β ₁₋₄₂ aggregation, reduced the insolubility and enabled detectable fluorescence of an A β ₁₋₄₂-GFP mutant¹⁸.

In the current study, we tried to visualize the molecular dynamics of wild type A β ₁₋₄₂ *in vivo* by arranging the length of linker sequence between A β ₁₋₄₂ and GFP in A β -GFP fusion proteins. Using this fusion protein, we revealed that A β ₁₋₄₂-GFP formed oligomers both *in vivo* and *in vitro*. The fusion proteins developed in this study are useful tools for screening candidate molecules of therapeutic drugs of AD and for investigating the function of intracellular oligomeric A β ₁₋₄₂ in cells.

Results

Visualizing A β -GFP fusion proteins in COS7 cells. Previous A β mutagenesis studies showed that the C-terminal fusion of A β ₁₋₄₂ to GFP prevents exact folding of the GFP protein in *Escherichia coli* (*E. coli*), thereby GFP does not fluoresce, whereas GFP fusion with a non-aggregating variant of A β ₁₋₄₂ showed retained GFP fluorescence¹⁸. To visualize the molecular dynamics of wild type A β ₁₋₄₂, we developed a new GFP fusion construct that fluoresces even when the fused A β ₁₋₄₂ proteins are aggregated. This construct, which derives the expression of human A β ₁₋₄₂ fused to the N-terminus of GFP, encodes a long linker sequence of 14 amino acids (QSTVPRARDPPVAT) between A β and GFP (Fig. 1A).

To observe the expression patterns of various A β -GFP fusion proteins, COS7 cells were transfected with the plasmids encoding A β -GFP (Fig. 1Ba), A β mut-GFP (Fig. 1Bb), or A β (E22 Δ)-GFP (A β bearing a mutation causing Alzheimer's disease; Osaka mutation, Fig. 1Bc). The A β mut-GFP protein contains F19S and L34P substitution, which were reported to suppress the aggregation of A β ₁₋₄₂¹⁸. In addition, COS7 cells were transfected with a GFP construct alone as a control (Fig. 1Bd). To confirm the expression of the A β proteins, transfected cells were immunostained with an anti- β amyloid antibody (6E10; Fig. 1Be–h), which recognizes all species of A β , i.e., monomer, oligomer, and fibril forms of it¹⁹. As shown in Fig. 1Bi–k, almost all GFP fluorescence in the cytoplasm of transfectants were colocalized with fluorescence of 6E10 antibody, except for cells transfected with GFP alone (Fig. 1Bl), indicating that GFP signals coincide with the localization sites of each A β fusion protein.

Cells transfected with GFP alone showed almost uniform GFP expression in the cytoplasm and nucleus (Fig. 1Bd,p), as also observed with A β mut-GFP transfected cells (Fig. 1Bb,n). In contrast, cells expressing the A β -GFP fusion protein showed aggregates of various sizes and shapes of A β -GFP in the cytoplasm (Fig. 1Ba,m), although the nuclear distribution appeared uniform. The expression patterns of the A β (E22 Δ)-GFP fusion protein were similar to that of the A β -GFP fusion protein, as aggregates of various sizes and shapes of A β (E22 Δ)-GFP were also observed throughout the cells (Fig. 1Bc,o).

To assess the polymerization states of A β -GFP fusion proteins inside of cells, each transfectant was immunostained using the 11A1 antibody, which was developed against E22P-A β ₁₀₋₃₅ as an antigen and recognizes oligomeric forms of A β specifically²⁰. Almost all GFP signals were double-labeled by the 11A1 antibody in A β -GFP transfected cells (Fig. 1C), suggesting that the A β -GFP aggregates were oligomer (Fig. 1Cg). However, the A β mut-GFP fusion proteins were only partially double-labeled with the 11A1 antibody, especially in the peripheral regions of the cell (Fig. 1Ch), indicating that most of the A β mut-GFP proteins do not form oligomers inside of cells. Immunoblot analysis following native-PAGE also supports the results of immunostaining. Non-denaturing protein lysates from COS7 cells that expressed each A β -GFP fusion protein were separated on a gel. We used anti-GFP and anti-A β (6E10) antibodies to detect the fusion proteins. A clear single band close to the GFP signal was detected in A β mut-GFP lysate by both antibodies. In contrast, smear and ladder-like signals were observed in both A β -GFP and A β (E22 Δ)-GFP lysates by these antibodies. These results indicated that most of the A β mut-GFP proteins exist as small-sizes molecules, probably monomers but A β -GFP and A β (E22 Δ)-GFP proteins exist as oligomers of different sizes in the cell (see Supplementary Fig. S1 and methods online).

We investigated the specific subcellular localization site of the A β -GFP fusion protein by double labeling with antibodies against marker proteins specific for mitochondria, Golgi apparatus, or endoplasmic reticulum, however, no double labeling was detected in those intracellular organelles (data not shown). To observe when and how the fusion proteins are expressed and accumulated in cells, we performed the time-laps imaging of COS7 cells transiently expressing A β -GFP (Supplementary Fig. S2). The A β -GFP fusion protein gradually aggregated in a time-dependent manner.

Comparison of GFP fluorescence intensity of A β -GFP fusion proteins according to the length of the linker sequence.

We considered that proper folding of GFP in fusion proteins may depend on the linker length between A β and GFP, and that the folding efficacy may affect the fluorescent intensity. To determine the effect of the linker length on the fluorescence intensities of fusion proteins, A β -GFP plasmids with a short-linker (0, 2, or 3 amino acid) or a long-linker (14 amino acids) were transfected into COS7 cells and rat hippocampal primary culture neurons, and the fluorescence intensities of the GFP fusion proteins were compared. Figure 2 shows images of cells expressing fusion proteins with a 2-amino acid linker (short-linker) or a long-linker. Twenty-four hours after transfection, both COS7 cells and primary neurons were immunolabeled by the 6E10 antibody, and confocal images were taken under the completely same condition as described in the “Methods” section. GFP fluorescence showed uniform cytoplasmic distribution in both cell types transfected with the long-linker construct, (Figs 1Ba and 2Ca). However, in cells transfected with the short-linker construct, GFP fluorescence was undetectable in the cytoplasm and was very faint in the nucleus (Fig. 2Ba,Cb), even though the immunolabeling signals of the 6E10 antibody were detected strongly (Fig. 2Bb,Cd). Comparison of the staining intensities observed with the 6E10 antibody and that of GFP fluorescence was performed in neurons expressing A β -GFP proteins with differing linker length (Fig. 2Cg,h). The immunofluorescence intensities observed with 6E10 were nearly identical for each fusion protein, but the GFP fluorescence intensities decreased as the length

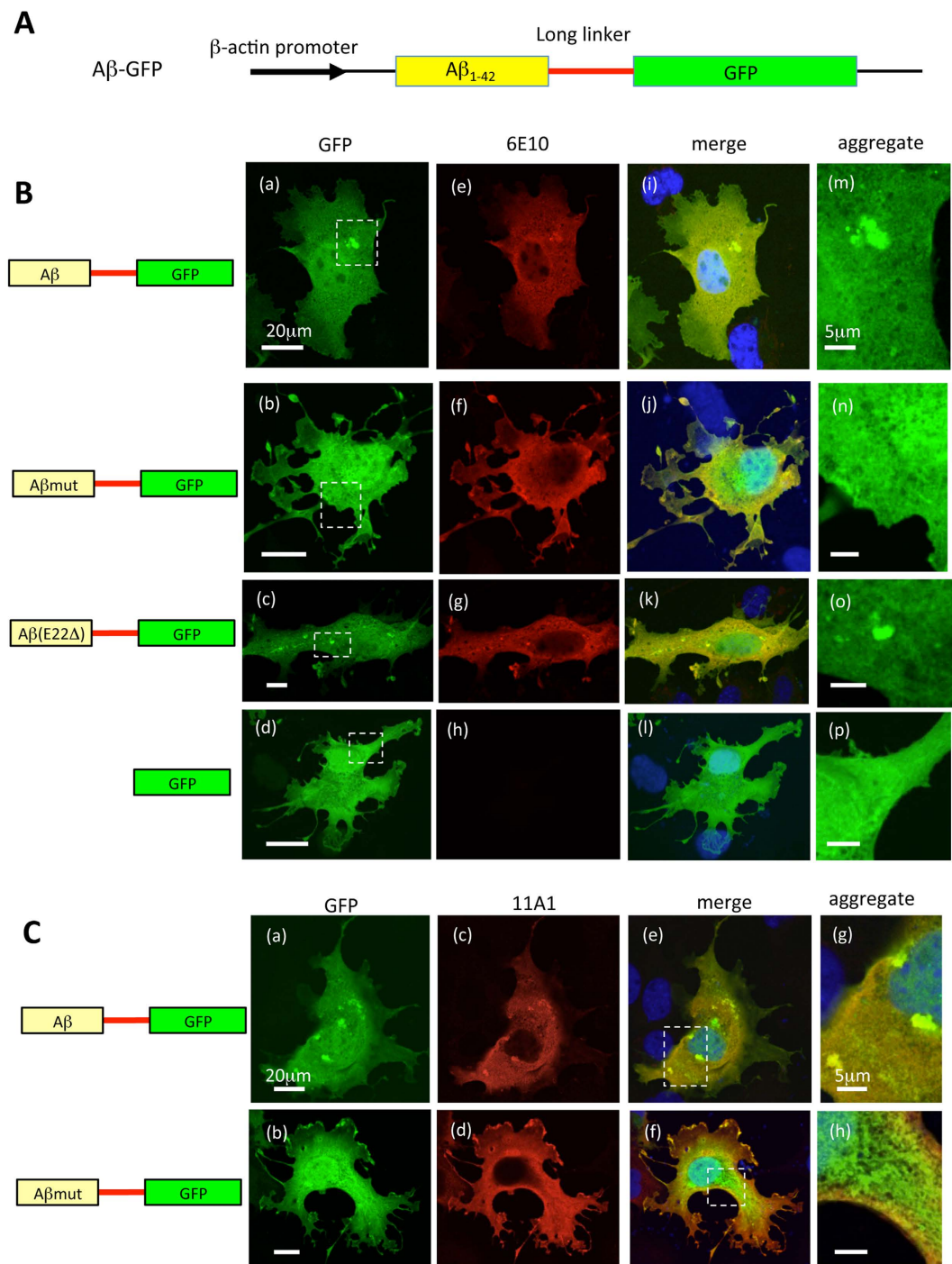


Figure 1. Representative images of COS7 cells transfected with various A β -GFP DNA constructs. (A) Basic structure of genes encoding fusion protein containing A β_{1-42} fused to GFP with a long-linker sequence (14 amino acids). (B) COS7 cells were transfected with plasmids encoding A β -GFP (a) A β mut-GFP (b) A β (E22 Δ)-GFP (c), or GFP (d). To confirm the expression of A β proteins, transfected cells were immunostained with the 6E10 antibody (e–h). Merged images with GFP are shown in (i–l). The regions within the dotted rectangles in (a–d) are enlarged in (m–p). Aggregated A β proteins (dotted localizations) were observed in A β -GFP and A β (E22 Δ)-GFP transfected cells, however, the A β mut-GFP proteins did not form detectable aggregates in cells. Scale bars: 20 μ m (a–d) 5 μ m (m–p). (C) Immunostaining of COS7 cells expressing the A β -GFP or A β mut-GFP fusion proteins with the 11A1 antibody. Merged images showed that almost all the A β -GFP fusion protein was labeled with the 11A1 antibody, indicating that the A β -GFP fusion protein formed oligomers. In contrast, the A β mut-GFP was only partially labeled with the 11A1 antibody. Scale bars: 20 μ m (a–f) 5 μ m (g,h).

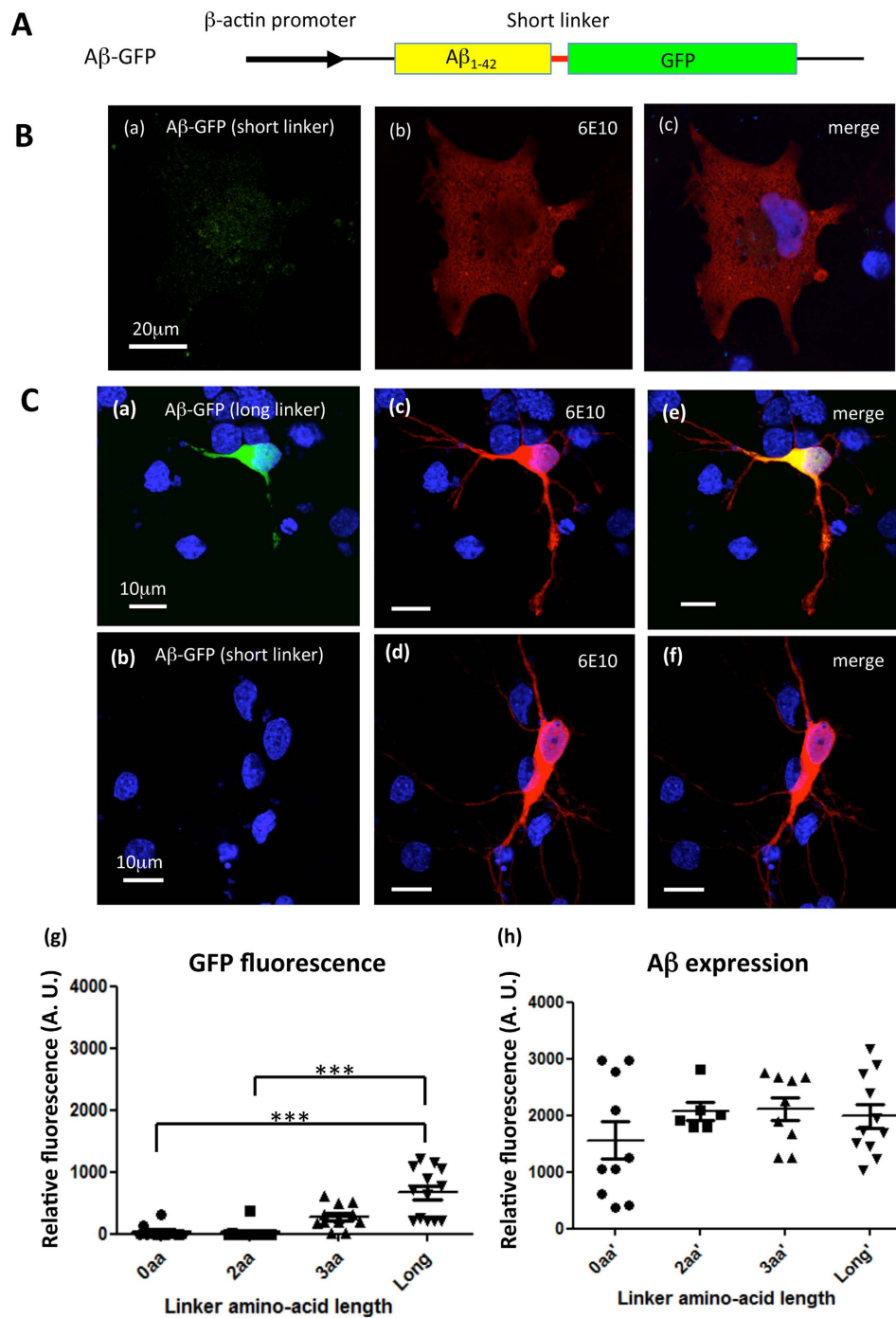


Figure 2. Comparison of A β -GFP fluorescence intensities according to the linker length in primary culture neurons. (A) Basic structure of genes encoding fusion proteins containing A β_{1-42} fused to GFP with short-linker sequences (0, 2 or 3 amino acids). (B) COS7 cells transfected with a short-linker A β -GFP (2 amino acids). Faint GFP fluorescence was detected in the nucleus and surrounding areas (a) even though the fusion protein was stained by the 6E10 antibody (b). Merged image of (a,b) is shown in (c). Scale bar: 20 μ m. (C) Primary culture of rat hippocampal neurons transfected with A β -GFP plasmids containing long-linker (a) or short-linkers (b). GFP fluorescence was nearly undetectable in cells carrying the short-linker plasmids, even though the fusion protein was stained by the 6E10 antibody (c,d). Merged images with GFP are shown in (e,f). Relative fluorescence intensities from cells expressing each fusion protein with various linker lengths were measured (g,h). Statistical analyses showed that the detection of the A β protein in neurons was nearly identical with each plasmid (h) but GFP fluorescence intensities increased significantly as the linker became longer (g) (***) ($p < 0.001$, Kruskal-Wallis test, $n = 10-14$ cells each). Scale bar: 10 μ m.

of the linker became shorter. These results indicated that GFP fused to A β via long-linker can fold normally and fluorescence robustly, whereas GFP cannot fluorescence robustly in the short-linker forms, probably because of misfolding.

Oligomerization of the A β -GFP fusion protein *in vitro*. To analyze the molecular characteristics of the A β -GFP fusion proteins in detail, we performed nuclear magnetic resonance (NMR) measurements and electron microscopy (EM) observations. We focused on the long-linker fusion proteins because only these proteins appeared to be folded normally. NMR spectra of synthetic peptide (Fig. 3A,E,F), A β -GFP (B), A β mut-GFP (C) and GFP (D) were collected to examine aggregation. We used Hou's method²¹ to generate the monomeric A β fusion proteins, as described in the "Methods" section. The NMR spectral intensities of the synthetic A β peptide decreased in a time-dependent manner and approached zero following approximately 8 h incubation period at 37 °C (Fig. 3A,E,F), indicating that nearly all peptides aggregated and formed fibrils. This is because the spin relaxation rate during ¹H NMR detection is inversely correlated to the overall rotational motion of the molecule²², resulting in impaired NMR signals to be observed for very large molecules, such as fibrils. For the A β mut-GFP, the spectra were unchanged even after a 63.5 h incubation at 37 °C (Fig. 3C), indicating that the monomeric state persists at 37 °C. The spectral intensity of the A β -GFP protein decreased by approximately 20% during the first 15.5 h incubation at 37 °C (green line), but not significantly during the subsequent 48 h (blue line, Fig. 3B). These data suggested that the aggregation stopped before fibril formation.

Next, we examined the molecular features of each A β -GFP fusion protein by negative-stain EM. The images of GFP showed round or rectangular particles ~3–4 nm in size (Fig. 4Aa), consistent with the atomic structure of GFP²³. Under unpolymerizing conditions for A β , the A β peptide was observed as smaller, round or elongated particles (Fig. 4Ab), possibly corresponding to single A β peptides. In the images obtained for A β -GFP, A β mut-GFP, and A β (E22 Δ)-GFP, some particles were only slightly larger than GFP, probably corresponding to single fusion proteins (Fig. 4Ac–e). Under the conditions that promote polymerization, the synthetic A β peptides formed fibrils of 7.34 ± 0.36 nm ($n = 30$) in width (arrowhead), or thicker filaments (arrow), which appeared to form following entwining of the fibrils with each other (Fig. 4Af). In contrast, A β -GFP, A β (E22 Δ)-GFP, and A β mut-GFP did not form regular fibrils. Instead, A β -GFP was observed as oligomers of various sizes (Fig. 4Ag) or as filamentous-looking aggregates (Fig. 4Ah), and A β (E22 Δ)-GFP was also observed as oligomers of various sizes (Fig. 4Aj). Magnified views of the dotted rectangles (inset of Fig. 4Ah,j) reveal that these aggregates are composed of small oligomeric clusters of ~10 nm. We call the single oligomeric cluster as 1 unit (arrows in inset). In the case of A β mut-GFP, however, these clusters were rarely observed and most of the molecules seemed to be in monomers or very small oligomers (Fig. 4Ai and magnified view of dotted rectangle in i).

We examined how many molecules were present in single units of A β -GFP and A β (E22 Δ)-GFP aggregates, or in a particle observed with A β mut-GFP. The estimated area of a single A β -GFP fusion protein was 13.7 nm². Therefore, the actual measured values of areas for each single unit of A β -GFP aggregates were divided by 13.7 nm². Figure 4B showed that the main species of single units of A β -GFP and A β (E22 Δ)-GFP fusion protein oligomers contained 2–4 molecules. The averaged number of molecules in one unit of A β (E22 Δ)-GFP seemed to be slightly larger than that of A β -GFP. The main species of A β mut-GFP was monomer to dimer, consistent with the idea that this mutation suppresses aggregation of A β . With all of the three fusion proteins, some small aggregates were observed, but long fibrils were not formed. Thus, both NMR and EM studies suggest that A β -GFP fusion proteins form small oligomers.

Fluorescence correlation spectroscopy (FCS) analysis of A β -GFP fusion protein in living cells.

To further confirm the oligomeric state of the A β -GFP fusion protein in living cells, we performed FCS analysis on cells expressing each fusion protein as well as on their lysates. First, we examined the properties of each fusion protein in aqueous solution, which were extracted from transfected COS7 cells. Compared to the diffusion constant for the GFP protein (111.0 $\mu\text{m}^2/\text{s}$), that of the A β -GFP fusion protein was significantly lower (74.8 $\mu\text{m}^2/\text{s}$, Cell lysate in Table 1), indicating that the protein mobility of the fusion protein was significantly decreased. The A β mut-GFP protein showed an intermediate diffusion constant (86.6 $\mu\text{m}^2/\text{s}$) between that of GFP and A β -GFP. Since the estimated molecular weight of single A β -GFP fusion protein is not markedly different from that of GFP (33 kDa vs 27 kDa), the decreased diffusion mobility observed with the A β -GFP fusion protein suggested that some molecular complex, presumably oligomers composed of several A β -GFP fusion protein molecules, are formed within cells. Interestingly, the count per molecule (CPM) value of the A β -GFP fusion protein was decreased compared to that of GFP, whereas that of A β mut-GFP showed similar value to that of GFP, suggesting that an increase in fluorescent intensity of a particle does not simply occur, even if the fusion protein forms oligomers (see the "Discussion" section).

We applied this observation directly to living COS7 cells and the results were similar to those obtained in aqueous condition (Live cell in Table 1). Because of restricted diffusion in the cell, autocorrelation functions were fitted using a two-component diffusion model for this analysis. The diffusion constant of A β -GFP in both the cytoplasm and nucleus was significantly lower than that of GFP, again suggesting decreased diffusion mobility due to the formation of larger molecular complex compared to GFP (estimated Mw; 27 kDa in GFP vs 77 kDa in A β -GFP. See the "Methods" section for calculation). The two-component diffusion model analysis applied for living cells also showed that remarkable decrease of fast component fraction in A β -GFP expressing cells (84%), compared to both GFP and A β mut-GFP (around 95%). This suggests that interactions between the A β -GFP protein and other intracellular species may be increased and/or the amount of large soluble aggregates formed by the A β -GFP protein may be increased within cells. The CPM value from cells expressing A β -GFP was decreased compared to that of GFP and A β mut-GFP, suggesting that fluorescence in the large soluble aggregates/oligomers may be quenched (see the "Discussion" section for further explanation). We have also applied this observation to examine the molecular dynamics of A β (E22 Δ)-GFP in cell lysate and in living cells. In both conditions, this

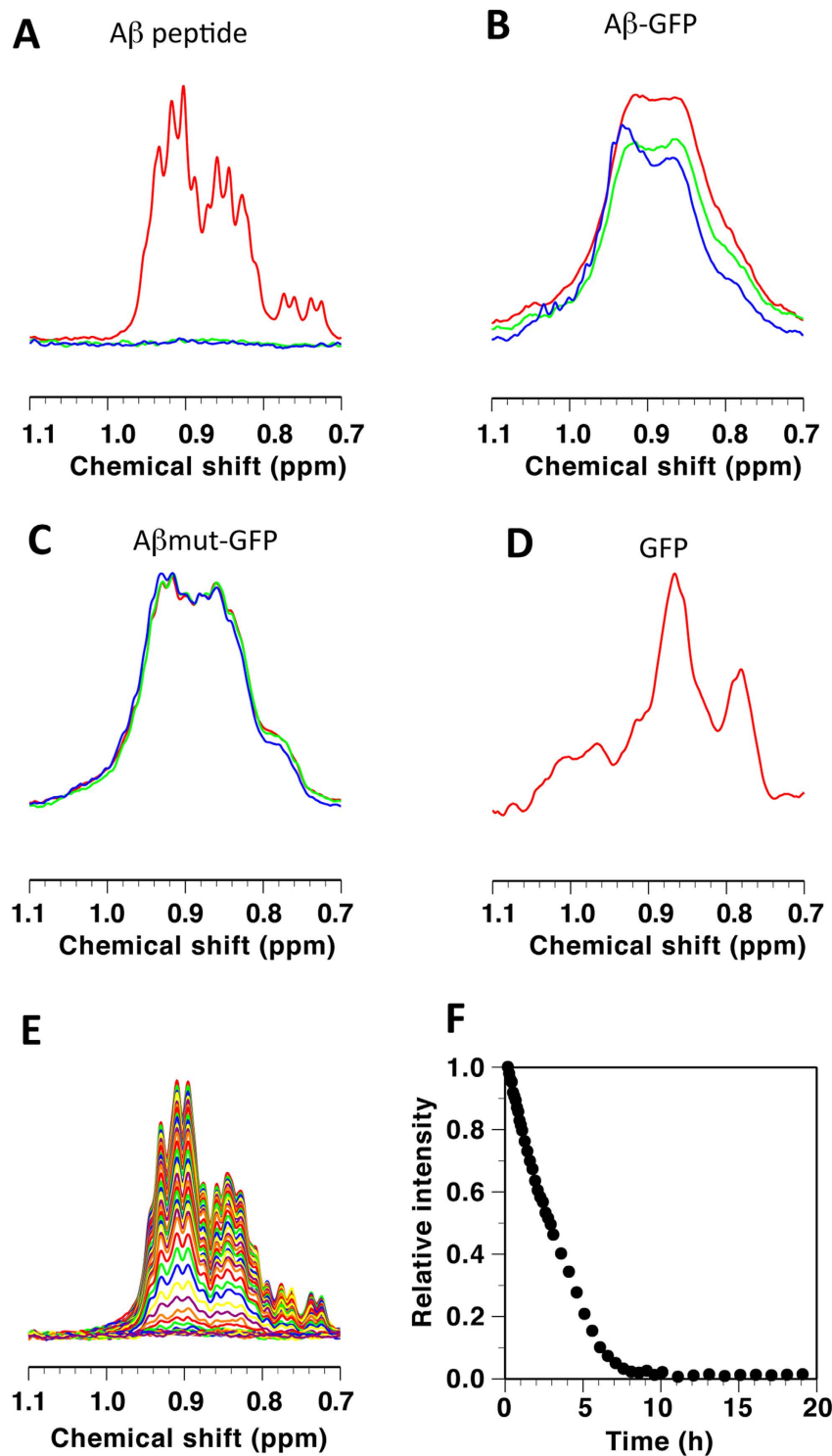


Figure 3. NMR analyses of structural changes in $A\beta$ -GFP fusion proteins. Shown are parts of 500-MHz NMR spectra mainly reflecting methyl groups for the $A\beta$ peptide (A,E), $A\beta$ -GFP (B), $A\beta$ mut-GFP (C), and GFP (D). Spectra in (A–D) were recorded at 20°C, where the red, green and blue lines indicate intact peptides, those after incubation at 37°C for 15.5 h, and those after incubation at 37°C for 50 h or 63.5 h, respectively. Spectra in (E) were recorded at 37°C after 5 min – 19 h of incubation, where changes in the intensity of the highest peak at 0.91 ppm are shown in (F).

mutant form showed significantly slower mobility than GFP, but the mobility was not significantly different from that of the wild-type $A\beta$ -GFP in any conditions. These results suggest that the E22 Δ mutation also causes the formation of large protein complex similar to the wild-type $A\beta$ -GFP. These FCS data confirmed that our fusion

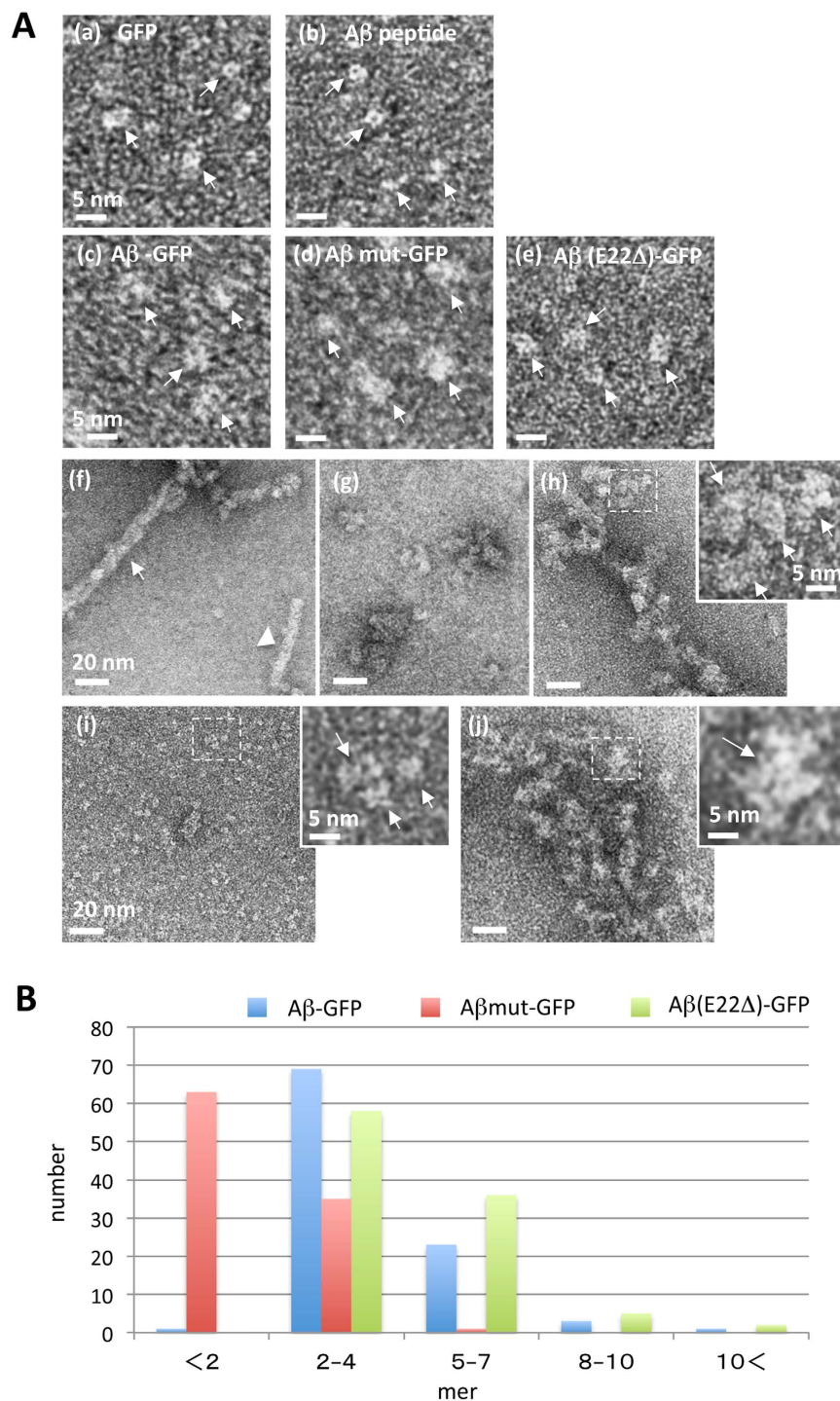


Figure 4. EM analysis of molecular feature of A β -GFP fusion proteins. EM images (A) and analyses (B) of A β -GFP fusion proteins. GFP (a), monomeric A β peptide (b), A β -GFP (c), A β mut-GFP (d), and A β (E22 Δ)-GFP (e) are indicated by arrows in each panel. 24 h after incubation at 4 °C (pH8.5), A β peptide formed long fibrils (f) but A β -GFP (g,h) and A β (E22 Δ)-GFP (j) formed oligomers with various sizes (g,j) or filamentous-looking aggregates (h). Almost all the A β mut-GFP remained as small particles in the size of a monomer or a very small oligomer (i) without a clear sign of polymerization. The inset shows a magnified view of the dotted rectangle in (h–j) revealing single units of A β -GFP fusion protein oligomers (arrows). Measurement of the area of each unit (B) shows that a single unit of polymerized A β -GFP and A β (E22 Δ)-GFP contains two to four molecules but the particles observed with A β mut-GFP contain single to two molecules ($n = 100$ units). Scale bars: 5 nm (a–e) and insets), 20 nm (f–j).

Protein expressed	N	CPM(kHz) ¹⁾	Fast (First) component		
			Diffusion constant ($\mu\text{m}^2\text{s}^{-1}$) \pm SEM ²⁾	Fraction (%)	Mw (kDa)
Cell lysate (Aqueous condition)					
GFP	5	1	111.0 \pm 1.4	-	27
A β -GFP	5	0.37 \pm 0.01	74.8 \pm 4.8 *	-	88
A β mut-GFP	5	0.89 \pm 0.11	86.6 \pm 1.9	-	57
A β (E22 Δ)-GFP	5	0.31 \pm 0.08	70.2 \pm 4.5 **	-	107
Live cell (Cytoplasm)					
GFP	28	1	60.8 \pm 3.0	96	27
A β -GFP	45	0.81 \pm 0.02	42.8 \pm 2.0***	84	77
A β mut-GFP	25	0.90 \pm 0.03	52.8 \pm 2.3	95	41
A β (E22 Δ)-GFP	27	0.65 \pm 0.03	34.9 \pm 2.6***	84	143
Live cell (Nucleus)					
GFP	27	0.93 \pm 0.03	52.8 \pm 1.5	95	27
A β -GFP	51	0.74 \pm 0.02	39.5 \pm 1.7***	83	64
A β mut-GFP	24	0.85 \pm 0.04	46.4 \pm 1.9 *	94	40
A β (E22 Δ)-GFP	27	0.68 \pm 0.01	43.9 \pm 3.1 *	81	47

Table 1. FCS analysis of A β -GFP proteins in living cells. ¹CPM values from lysate samples are normalized by that of GFP, and live-cell CPMs are normalized by that of GFP in cytoplasm. ²Kruskal-Wallis and post-hock tests are performed among each condition. Only the significant differences are shown: ***P < 0.001, **P < 0.01, *P < 0.05.

proteins generated by a long-linker sequence showed robust fluorescence and can be used to monitor the molecular dynamics of A β containing various types of mutations.

Expression of A β -GFP fusion proteins in transgenic *C. elegans*. Both the *in vitro* analyses of the molecular state of A β -GFP fusion proteins and the *in vivo* analyses of living cultured cells suggested that the fusion proteins probably exist as oligomers. These results also indicated that the fluorescence of the fusion proteins can be altered dependent on their aggregation properties when a short-linker is used. To examine whether these phenomena can also be observed in neuronal cells of a living animal, we expressed our fusion proteins in *C. elegans* neurons and observed their dynamics *in vivo*. A schematic representation of the A β -GFP fusion construct used for transgenic *C. elegans* strains is shown in Fig. 5A. A β -GFP was specifically expressed in the cholinergic neurons by the *acr-2* promoter. GFP fluorescence was detected steadily inside of the neurons in GFP transgenic animals (Fig. 5Ba). In transgenic animals expressing long-linker A β -GFP, GFP fluorescence was observed in both the cell bodies and their neurites, but showed accumulated or aggregated expression patterns of the fusion protein (Fig. 5Bb). However, GFP fluorescence was absent in the short-linker A β -GFP transgenic worms (Fig. 5Bc), which is similar to the expression patterns observed in COS7 cells and rat hippocampus primary neurons (Fig. 2).

We also wondered whether the fluorescence intensities in transgenic animals expressing short-linker A β -GFP reflect the aggregation properties of fusion proteins. To examine this, we expressed A β mut-GFP fusion protein with the short-linker, and GFP fluorescence was clearly and uniformly detected in the neuronal cells of A β mut-GFP transgenic worms (Fig. 5Bd). This finding indicates that non-fibril and soluble forms of A β do not affect the folding of GFP and that GFP fluorescence can be observed in living neurons if aggregation of the fusion protein is inhibited.

Therefore we examine whether these phenomena could be used to screen for substance that inhibit A β aggregation. It is known that curcumin can inhibit polymerization of A β . Thus we added it to the culture medium and the molecular state of short-linker forms of A β -GFP was observed in transgenic worms. In the animals reared on

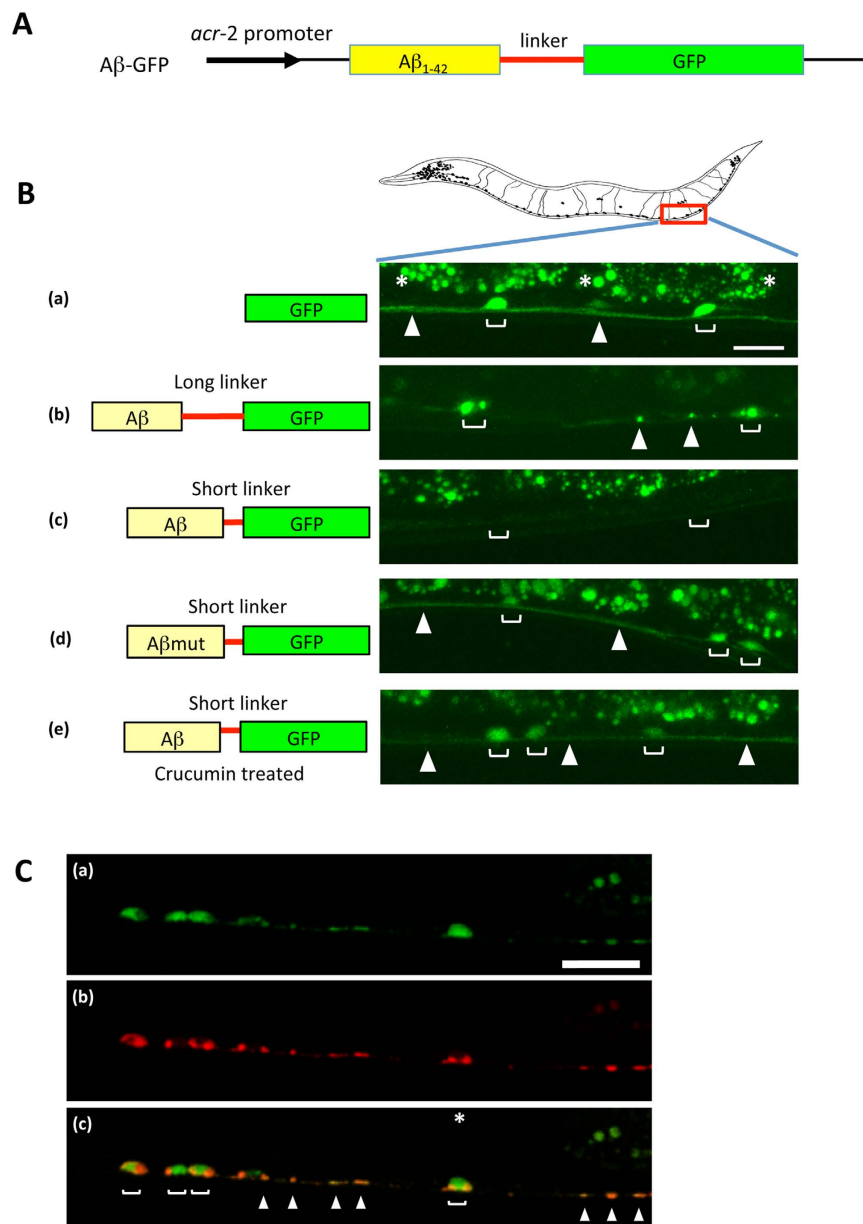


Figure 5. Expression of A β -GFP fusion proteins in *C. elegans*. (A) Schematic representation of the A β -GFP fusion construct. (B) GFP fluorescence in the cholinergic motor neurons of A β -GFP transgenic *C. elegans*. The left illustration depicts the expressed proteins shown in the right pictures. The right pictures show the expression patterns of fusion proteins in *C. elegans*. (a) GFP, (b) A β -GFP with a long-linker, (c) A β -GFP with a short-linker, (d) A β mut-GFP with a short-linker, and (e) curcumin treatment of animals bearing a short linker protein. Blankets indicate the cell bodies of neurons and arrowheads indicate the axon in the ventral nerve cord. Asterisks indicate the autofluorescence from the intestine. The long-linker has 14 amino acids and the short-linker has only 2 amino acids sequences. Cells expressing the short-linker A β -GFP protein did not show fluorescence (c) but the long-linker one and A β mut-GFP showed bright fluorescence (b,d). Short-linker A β -GFP transgenic *C. elegans* were treated with curcumin, which induces A β disaggregation. Disappeared fluorescence was recovered after treatment with curcumin (e). Scale bar: 10 μ m. (C) Localization of the A β -GFP fusion protein at the presynaptic regions. A β -GFP (a) and presynaptic protein SNB-1 fused with mCherry (b) were simultaneously expressed in cholinergic neurons. Several GFP puncta were co-localized with SNB-1 on the axon (c) suggesting that the fusion protein may be strongly accumulated at synaptic sites. Scale bar: 10 μ m.

plates containing curcumin, bright and uniform GFP fluorescence was observed in both cell bodies and neurites, similar to animals expressing the A β mut-GFP protein (Fig. 5Be). These findings indicated that the inhibition of A β aggregation induced by curcumin results in the recovery of GFP fluorescence.

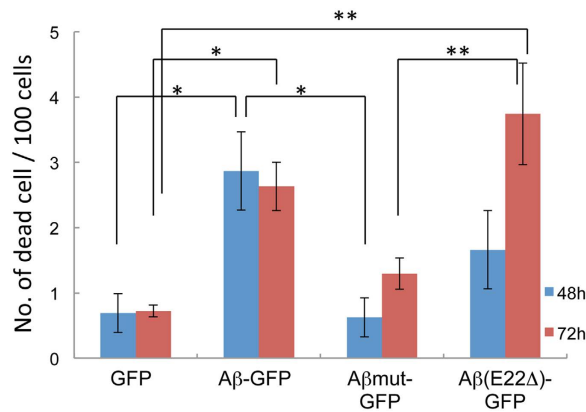


Figure 6. Effect of A β -GFP fusion proteins on the survival of COS7 cells. The numbers of dead COS7 cells were counted 48 h (blue) and 72 h (red) after transfection with plasmids encoding GFP, A β -GFP, A β mut-GFP, or A β (E22 Δ)-GFP. The number of dead cells increased significantly in A β -GFP and A β (E22 Δ)-GFP transfected cells compared with the A β mut-GFP and GFP control transfected cells. The data represents the mean \pm SEM (100 cells), * p < 0.05, ** p < 0.001 by one-way ANOVA.

This fusion protein can be also used to examine the subcellular localization of A β protein (Fig. 5C). The presynaptic VAMP2 protein (SNB-1 in *C. elegans*) was fused to mCherry and simultaneously expressed with the long-linker A β -GFP fusion protein, under the control of the same promoter. Several strong accumulations of the A β -GFP fusion protein correlated well with the position of RFP localization, meaning that the fusion protein tended to accumulate at the synaptic regions when the protein is expressed in presynaptic neurons.

Effect of A β -GFP oligomers on survival rate of COS7 cells. To examine whether the A β -GFP fusion protein caused cellular toxicity in living cells, we measured cell death ratios in COS7 cells transfected with each A β -GFP fusion plasmid or the GFP plasmid (Fig. 6). Compared with cells expressing GFP, the ratios of dead cells significantly increased in both A β -GFP and A β (E22 Δ)-GFP transfected COS7 cells until 72 h after transfection, but it was not changed in A β mut-GFP expressing cells. These results indicate that A β -GFP and A β (E22 Δ)-GFP oligomer may cause cellular toxicities like wild-type A β oligomers.

Discussion

The intracellular accumulation of A β_{1-42} has been proposed as an event responsible for early pathogenesis of AD. Especially, A β oligomer has been the subject of much attention as a target for studying the pathophysiological role of AD^{24,25}, because it has been proposed to be a key mediator of cognitive decline in AD¹¹. In this study, we developed new cellular and animal models of AD, which showed an accumulation of small sized A β oligomers inside of cells. This molecular state can be achieved by fusing A β and GFP, and this method can be used to visualize the molecular dynamics of A β in living cells by arranging the linker sequence between A β and GFP.

Previous report using yeast lysate that expresses A β -GFP fusion proteins suggested that A β_{1-40} -GFP and the A β -GFP mutant that contains substitution Ile 41 to Glu and Ala 42 to Pro are less prone to aggregation and a portion of those fusion proteins exhibit soluble and non-aggregated forms, but A β_{1-42} -GFP exhibit insoluble aggregate only²⁶. We confirmed the molecular features of GFP-fused A β proteins through several strategies. In NMR experiments, we started the measurement for all samples under monomer conditions and the same concentration of proteins. Previous findings indicated that GFP is stable at pH 6–10^{27,28} and that NaOH does not affect the conformational, tinctorial, morphological, and physiological functions of A β ²⁹. Therefore, our methods to form monomers should not affect the protein properties of A β -GFP fusion proteins. Our results indicated that the synthetic peptide formed fibrils within 8 h incubation period, however, the multimerization of A β -GFP proteins stopped before 15.5 h, consequently they could not form fibrils and remained as oligomers. In the EM experiments, the synthetic peptides formed long fibrils, but A β -GFP formed oligomers consisting of mainly 2–4 molecules, which did not assemble further into fibrils or large aggregates. These results were consistent with the NMR results and showed that A β -GFP form oligomers *in vitro*. The fusion protein is composed of a 27 kDa GFP component and a 4.5 kDa A β , thus a GFP molecule is much larger than the A β molecule. Therefore, aggregation of A β might be sterically hindered by GFP and, as a result, A β -GFP fusion protein could form only oligomers.

FCS analysis also suggested that the same molecular states of A β -GFP fusion proteins exist in living cells. In cultured living cells, the estimated molecular size of A β -GFP calculated from the diffusion constant was 77 kDa in the cytoplasm and 64 kDa in the nucleus. The larger molecular sizes probably result from either the formation of oligomers or molecular complexes with intracellular proteins. Contrary to the slow diffusion mobility of the A β -GFP fusion proteins, their CPM values, which refer to fluorescence intensities per single particle, were smaller than that of GFP as well as a non-aggregating mutant, A β mut-GFP (Table 1). These results suggest that homo-oligomeric species of A β -GFP may emit low fluorescence intensity because of quenching of GFP fluorescence. By fusing A β_{1-42} to the N-terminus of GFP, the folding properties of GFP could be altered, and only a small fraction of the fusion proteins can express fluorescence^{17,18}. Due to this fusion protein's nature, the CPM values of the A β -GFP fusion protein may not appear to correspond to the multimerization state of the fusion protein.

Based on this argument, we focused on the values of diffusion constant and estimate that the major forms of oligomers of the A β -GFP protein may be trimers to tetramers in live cells. In contrast, A β mut-GFP showed a fraction of the fast component similar to that of GFP, but the estimates molecular weight (41 kDa) was between that of a monomer and a dimer, suggesting that the fusion protein probably exists as a mixture of monomers and dimers. The molecular state of the A β (E22 Δ)-GFP mutant did not show a clear difference in molecular mobility from the wild-type A β -GFP, although it causes AD and shows an age-dependent intraneuronal accumulation of oligomers¹⁶. We believe that, however, our methods enable us to understand the intracellular dynamics of these kinds of mutant proteins of A β . Further investigations of appropriate linkers to generate A β -GFP with a less quenching property will clearly improve detection sensitivity of the A β oligomers in live cells using FCS. In COS7 cells transfected with the A β -GFP plasmid, almost all A β -GFP fusion proteins were labeled with the 11A1 antibody, which recognizes oligomeric A β specifically²⁰. The decrease of the fast fraction in A β -GFP (84% and 83%) also means that larger complexes probably exist in living cells. Taken together, these data suggested that A β -GFP fusion proteins formed small oligomers inside cells and that this fusion protein is a new useful tool for assessing the intracellular function and toxicity of the A β oligomers.

Previously, it was reported that insoluble aggregates of N-terminal fusion partners with GFP attenuated the fluorescence of GFP¹⁷. A linker was constructed using 12 amino acids to make a reporter construct for evaluating protein folding, which was designed to avoid large bulky hydrophobic residues. Wurth *et al.*¹⁸ used the same construct to make a fusion protein composed of a N-terminal GFP fused to C-terminal end of A β ₁₋₄₂. In this case, the wild type A β -GFP fusion protein did not fluoresce in *E. coli* whereas strong fluorescence was observed in the mutated A β -GFP fusions containing substitutions in the hydrophobic region responsible to aggregation of A β . Nair *et al.*²⁶ also reported using mutant A β -GFP fusion constructs with which aggregation is reduced that the fluorescence intensity in yeast reflects the aggregation state of A β . In the current study, we inserted 14 random amino acids as a linker sequence between A β and GFP (long-linker), and succeeded in developing new A β -GFP fusion proteins that fluoresce even when the wild type A β is aggregated. We do not fully understand the exact reasons why this linker sequence can generate stable fluorescence in an aggregated condition. Using this construct, however, it is possible to observe the molecular dynamics of A β -GFP oligomers in living cells. There are some reports suggesting that A β provokes ER stress and oxidative stress and induces cellular toxicities³⁰⁻³⁵. In particular, the E22 Δ mutant caused ER stress, which induced apoptosis in HEK293 cells³⁶. We also found that both A β -GFP and A β (E22 Δ)-GFP caused increased cell death in transfected COS7 cells. These findings indicated that GFP-tagged A β fusion proteins may have similar physiological properties as wild type A β suggesting that they are quite applicable for examining “oligomer hypothesis” in AD. From our EM and FCS results, the molecular states of wild type A β -GFP and A β (E22 Δ)-GFP are not greatly different from each other. Therefore there is a possibility that larger aggregates have a potential to induce strong toxicity compared to smaller aggregates, but the toxicity of these fusion proteins against cells may not depend solely on the aggregation sizes of A β . Although the difference in the structures of these fusion proteins are not known, there might be unknown interactive factors with A β oligomers that induce the toxicity inside cells.

To evaluate the property of our fusion proteins in living animals, we used *C. elegans* as an experimental model and observed *in vivo* A β dynamics. Although invertebrate is phylogenetically far removed from mammals, *C. elegans* possesses several genes homologous to the human AD-related genes such as nicastrin³⁷, presenilin^{38,39}, APH-1⁴⁰ and neprilysin⁴¹. In addition to these genetic relationships, over expression of A β exhibits an increased level of reactive oxygen species (ROS) in *C. elegans*⁴² similar to those observed in AD patients. In *C. elegans* neurons, we confirm that our fusion proteins showed fluorescence properties quite similar to those in mammalian cells including rat primary cultured hippocampal neurons and COS7 cells, i.e., the protein with the short linker decreases its fluorescence when it aggregates, whereas the long linker retains fluorescence in spite of its aggregation. Therefore, *C. elegans*, as well as mammalian models, is a useful tool to investigate the basic pathogenesis of AD. Using this model system, we can perform both the genetic and chemical screening for endogenous or exogenous factors that are possible to regulate the A β aggregation in live animals. For example, the curcumin experiment in short-linker A β -GFP transgenic worms revealed that it is possible to monitor the effect of drugs against deposition and disaggregation of A β directly in living animals, suggesting that our new fusion proteins are useful tools for screening candidate synthetic chemical and naturally occurring products against AD in living neuron.

Methods

Plasmids construction. To generate the A β ₁₋₄₂-GFP expression vector, we amplified A β ₁₋₄₂ from a plasmid containing the human APP coding sequence (DNAFORM, clone ID:100068486) with a 5' primer including a *Hind*III site and 3' primer including a *Sal*I site. The resultant PCR fragment was subcloned into a modified pEGFP-N1 vector (Takara BIO Inc, Shiga, Japan) containing a chicken β -actin promoter⁴³ to generate the pAct-A β ₁₋₄₂-GFP plasmid. Expression vectors encoding A β ₁₋₄₂-GFP proteins with short-linkers, which include 0, 2(LE), or 3(LET) amino acids of the linker sequence between A β ₁₋₄₂ and GFP, were generated by deleting the linker sequence from the pAct-A β ₁₋₄₂-GFP plasmid.

Mutant forms of A β -GFP were generated using GeneArt® Site-Directed Mutagenesis System (Life Technologies) with the pAct-A β ₁₋₄₂-GFP plasmid serving as a template. The A β mut-GFP is a mutant generated by substituting Phe19 with Ser and Leu34 with Pro. The A β ₁₋₄₂(E22 Δ)-GFP is a deletion mutant constructed by removing glutamate-22 of A β ₁₋₄₂ sequence from the pAct-A β ₁₋₄₂-GFP plasmid.

To express A β -GFP fusion genes in *C. elegans*, the human A β ₁₋₄₂ coding sequence was inserted into the Fire's *C. elegans* GFP expression vector (a kind gift from A. Fire). A 3.0 kb upstream region of the *acr-2* gene was used to specifically express the fusion proteins in cholinergic motor neurons. The same promoter region was inserted into the mCherry vector to generate a *Pacr-2: snb-1: mCherry* fusion construct. The *C. elegans snb-1* cDNA fragment was amplified by RT-PCR and was subcloned in frame into the *Pacr2: mCherry* vector. All plasmid DNAs were sequenced, and the sequences are available on request.

Generation of transgenic *C. elegans*. Transgenic worms were generated using standard microinjection methods for *C. elegans*. The pBLH98 [*lin-15(+)*] or *Pacr-2::RFP* plasmid was used as a co-injection marker at 50 ng/ μ l. Each fusion plasmid was injected at 10–25 ng/ μ l. At least 3 independent stable transgenic lines were used in the experiments performed in this study.

Cell culture and transfection. All experimental procedures performed on animals were carried out in accordance with the approved guidelines in ethical permit approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology (Permission No. 2014-143) and in accordance with the Law No. 105 passed by and the Notification No. 6 released by the Japanese Government.

Primary cultures were prepared from the hippocampi of embryonic day 17 Wistar rats (Nihon SLC, Shizuoka, Japan). Briefly, embryos were removed by cesarean sectioning, after which hippocampi were isolated and digested in 1 U/ml papain (Worthington Biochemical corporation, NJ, USA) in HEPES-buffered Hank's Balanced Salt Solution containing cysteine and BSA (1 mg/ml each) at 37 °C for 12 min. Dissociated cells were plated on poly-L-lysine coated glass cover slips at a density of 30,000 cells/well in a 6-well plate in plating medium containing Dulbecco's Modified Eagle Medium (DMEM, Wako, Osaka, Japan) and 10% fetal bovine serum (FBS, Gibco, NY, USA). Two hours after plating, the medium was changed to Neurobasal[®] Medium (Invitrogen, CA, USA) containing B27 supplement (Invitrogen) and 0.5 mM L-glutamine.

COS7 cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂/95% air. For transfection of plasmid DNAs, cells were plated at a density of 15,000 cells/cm² in a 6-well plate.

COS7 cells and 4DIV primary hippocampal neurons were transfected with plasmids using Lipofectamine 2000 (Invitrogen), as described previously⁴⁴. Dead cells were counted 48 h and 72 h after transfection by staining the living COS7 cells with DAPI. The DAPI solution (Dojindo, Kumamoto, Japan) was added to the medium of each transfected cells and incubated for 20 min. After washed by PBS, five images were taken from each culture dish, and cells stained by DAPI were counted as dead. The total number of cells in images were also counted and used to calculate the number of dead cells per 100 cells. Statistical analysis was performed by one-way analysis of variance (ANOVA).

Immunohistochemistry and imaging of cells. To confirm the expression of each A β -GFP fusion protein, transfected COS7 cells and primary neurons were immunostained with an anti- β amyloid (6E10) antibody (Covance, WI, USA) or 11A1 antibody (IBL, Gunma, Japan). Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 15 min at room temperature. Then, the cells were washed with PBS and permeabilized for 5 min in 0.25% Triton X-100. After washing the cells in PBS, they were blocked in 3% normal goat serum (Vector Laboratories, Inc., CA, USA) for 30 min and incubated with the 6E10 antibody (\times 800 dilution) or 11A1 antibody (\times 50 dilution) overnight at 4 °C. Each protein was visualized with an Alexa 568 conjugated secondary antibody (Invitrogen), and the nuclei were labeled with DAPI (Vector Laboratories, Inc.). Cell imaging was performed using Olympus FluoView 1000 (Olympus, Tokyo, Japan) and Nikon A1R (Nikon, Tokyo, Japan) confocal laser scanning microscopes. Each image was obtained at a resolution of 1020 \times 1020 pixels. To quantify fluorescence intensities, the confocal scanning settings of pinhole, laser power, brightness, and contrast were held constant for all images. The fluorescence intensities of cell bodies were measured using Image J software (NIH, Bethesda, MD, USA). Statistical analysis of fluorescence intensities was performed by the Kruskal-Wallis test ($n = 10$ –14 cells each).

Time lapse imaging was performed using COS7 cells transfected with A β -GFP plasmid containing long-linker. Eighteen hours after transfection, fluorescent images of cells were taken every 10 min for 24 h in a humidified atmosphere containing 5% CO₂/95% air at 37 °C using a \times 20 objective lens (KEYENCE corporation, Osaka, Japan).

Curcumin treatment of A β -GFP transgenic *C. elegans*. Curcumin were dissolved in ethanol, and 100 μ l of 3 mM solution was spread onto NGM plates. Young-adult transgenic worms expressing short-linker A β -GFP protein were placed on the curcumin plates, and the fluorescence of their progeny was examined.

Purification of the A β -GFP fusion protein. Purifications of recombinant proteins were performed according to the manufacturer's instructions (New England BioLabs, MA, USA) and Chong *et al.*⁴⁵. Briefly, the coding sequences of A β -GFP, A β mut-GFP, and GFP were cloned into the pTXB1 vector (New England BioLabs) and the resulting plasmid DNAs were transformed into *E. coli* BL21 cells. The cells were grown in LB media at 37 °C until the culture reached an OD_{600 nm} of 0.5, and then the cells expressed the fusion protein by adding of 0.2 mM IPTG and incubated at 30 °C for 4 h. Cells were harvested and resuspended in Tris buffered solution (buffer A: 20 mM Tris-HCl, pH 8.5, 300 mM NaCl and 10% glycerol). After adding 0.002% CHAPS, 0.05 mM EDTA (pH 8.0) and 0.1 mM PMSF, the cell suspensions were incubated for 30 min at 4 °C and then ultrasonic disruption were performed on ice, using a BRANSON SONIFIRE 250. The lysed cell suspensions were centrifuged at 9600 \times g for 20 min at 4 °C, after which the supernatants were loaded onto equilibrated Chitin beads (New England BioLabs) and incubated for 1 h on rotator at 4 °C. The beads were loaded in a column and washed with buffer A, and then the fusion proteins were eluted by Tris buffered solution (buffer B: buffer A containing 50 mM DTT) for 16 h at room temperature. The concentrations of proteins were measured using the BCA protein assay kit (Pierce). Purified fusion proteins were stored at -80 °C until use.

NMR analysis. To produce the monomeric A β -GFP and A β mut-GFP, 1 N NaOH were added to the purified protein solution until the pH reached 10–11, and then the protein solution was sonicated for 1 min²¹. Synthetic A β peptide (Peptide Institute, Inc. Osaka, Japan) was predissolved in 10 mM NaOH solution at a concentration of 500 μ M. Then, the buffer of each protein was displaced to 20 mM deuterated Tris-HCl (d₁₁, 99%; CDN isotope,

QC, Canada) at pH 7.2 containing 0.05 mM NaN₃ by ultrafiltration. After centrifugation at 15,000 × g for 5 min, the solutions were kept at 4 °C. Purified GFP was also subjected to the above process. Immediately before the NMR measurements, solutions of 25 μM protein/peptide were dissolved in 20 mM deuterated Tris-HCl (pH 7.2) containing 0.1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate and 5% D₂O (Cambridge Isotope, MA, USA).

NMR measurements were performed on a Bruker Avance III-500 spectrometer at 20 °C. Water suppression was achieved by the WATERGATE method⁴⁶. In addition to the monomeric proteins/peptides, those incubated at 37 °C for 15.5 h or 50–63.5 h were subjected to the measurements. Also, successive NMR measurements of the Aβ peptide solution at 37 °C for a real-time monitoring of aggregation were carried out.

Electron microscopic analysis. Monomeric and polymerized fusion proteins were observed by EM. Monomeric fusion proteins were prepared as described above. Polymerizations were performed by incubating the monomers at 4 °C for 24 h in buffer A. Synthetic peptides (generous gifts from Dr. Miyagishi, AIST) were pre-dissolved in NH₃ and polymerized in buffer A at a × 1000 dilution. For the EM observations, each sample was diluted to a final concentration of 0.2 μM.

Small aliquots (5 μl) of samples were deposited onto carbon coated EM grids and negatively stained with 1% uranyl acetate, as described⁴⁷. After blotting away the excess solution and air-drying, observations were performed using a Tecnai F20 EM (FEI) operated at 120 kV. The images were recorded with an ORIUS SC600 slow-scan CCD camera at a magnification of × 80,000.

Length, diameter, and area measurement were performed using Image J software. The areas were used to evaluating how many single molecules were polymerized to form a single unit of Aβ-GFP fusion protein oligomers. The observed areas of the oligomers were divided by that of the monomeric Aβ-GFP fusion protein. The area of a single GFP protein is ~12 nm², based on the dimensions of the atomic structure of a GFP molecule (~3 nm × 4 nm)²³, and the observed images agreed with the prediction (see Results). Since the ratio of the molecular weights of GFP : Aβ : Aβ-GFP (including the linker sequence) is ~27 : 5 : 33, we estimated that the ratio of the area would be roughly 9 : 2.9 : 10.3, so that the area of a monomeric Aβ-GFP is ~13.7 nm².

FCS measurement. FCS measurements were performed as described previously^{48–50}. For the *in vivo* analysis of living cells, COS7 cells were cultured on 35 mm glass base dish (AGC Techno Glass, Shizuoka, Japan), and transfected with GFP or Aβ-GFP plasmids at 17–27 h before analysis. FCS measurements in live cells were performed for both the cytoplasmic and nucleus regions in the same transfected cells, using an LSM 510 META confocal microscopy equipped with ConfoCor3 (Carl Zeiss, Jena, Germany). GFP was excited at 488 nm using an Argon ion laser through a C-Apochromat 40 × 1.4NA Korr. UV-VIS-IR water immersion objective. For the analysis of cell lysates, transfected COS7 cells were once washed in ice cold PBS and lysed in CellLytic M (Sigma-Aldrich, MO, USA) containing cOmplete Protease Inhibitor cocktail (Roche Diagnostics, Basel, Germany). After a 15 min incubation on shaker at 4 °C, the cells were harvested and their supernatants were recovered after centrifugation (12,000 × g) for 15 min. FCS measurements from cell lysates (aqueous solution) were performed using the Hamamatsu FCS Compact system (Hamamatsu Photonics, Shizuoka, Japan). Fluorescence autocorrelation functions in cell lysates were measured for 30 sec and analyzed using an one-component model, while those in living cells were measured for 60 sec and fitted using a two-component diffusion model. In both analyses, the structure parameters were determined using rhodamine 6 G (Rh6G) and were set from 4 to 6. The diffusion constant of fusion proteins (D_{sample}) was calculated as follows:

$$D_{\text{sample}} = (\tau_{\text{Rh6G}}/\tau_{\text{sample}}) \cdot D_{\text{Rh6G}} \quad (1)$$

where τ_{Rh6G} and τ_{sample} are the diffusion time of Rh6G and the sample, respectively, and D_{Rh6G} is the given diffusion constant of Rh6G (414 μm²/s). CPM values were normalized using that of GFP observed in the same experimental set. The molecular weight (MW) of the fusion proteins was calculated using following equation modified from Stokes-Einstein relation:

$$MW_{\text{sample}} = MW_{\text{GFP}} \cdot (D_{\text{GFP}}/D_{\text{Sample}})^3 \quad (2)$$

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Author Contributions

T.O., M.D., T.E. and H.S. designed the research; T.O., M.D., K.Y., K.H. and A.K. performed the research; T.O., M.D., K.Y., K.H., A.K., T.U., N.H., M.K., T.E. and H.S. analyzed the data; and T.O., M.D., K.Y. and K.H. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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