



Mini Review

Cell-based Models To Investigate Tau Aggregation

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ABSTRACT

Accumulation of abnormal tau aggregates in neuron is an important pathological signature in multiple neurodegenerative disorders including Alzheimer's disease. Tau is a neuron specific microtubule-associated protein that regulates microtubule stability, which is critical for axonal outgrowth and synaptic plasticity. In a pathological condition, tau dissociates from microtubules and forms insoluble aggregates called neurofibrillary tangles (NFTs). The accumulation of NFTs in neuron directly correlates with microtubule dysfunction and neuronal degeneration. Due to the pathophysiological importance of tau, great efforts have been made to understand tau aggregation processes and find therapeutics to halt or reverse the processes. However, progress has been slow due to the lack of a suitable method for monitoring tau aggregation. In this mini-review, we will review the conventional methods for studying tau aggregation, and introduce recent cell-based sensor approaches that allow monitoring tau aggregation in living cells.

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1. Introduction

Tau is a neuron specific microtubule-associated protein [1–3]. In a healthy neuron, tau binds to microtubules and regulates microtubule stability, which is critical for axonal outgrowth and neuronal plasticity [4–6]. When pathologically altered, tau molecules are not able to stabilize microtubules and become insoluble aggregates [3,7–9]. Since Alois

Alzheimer discovered the abnormal tau inclusions in a patient's brain, the presence of tau aggregates is a critical biomarker for making the pathological diagnosis of AD [10]. In AD patients, three forms of tau aggregates occur; neurofibrillary tangles (NFTs) in neuronal somata, neuropil threads (NTs) in neuronal dendrites, and neuritic plaques (NPs). These tau aggregates induce neuronal degeneration. Especially, the density of NFTs correlates fairly well with regional and global aspects of cognitive decline during the progression of AD [10]. Hence, there has been great effort to understand how the deposition of NFT causes neurodegeneration (Fig. 1). NFT may damage neurons and glial

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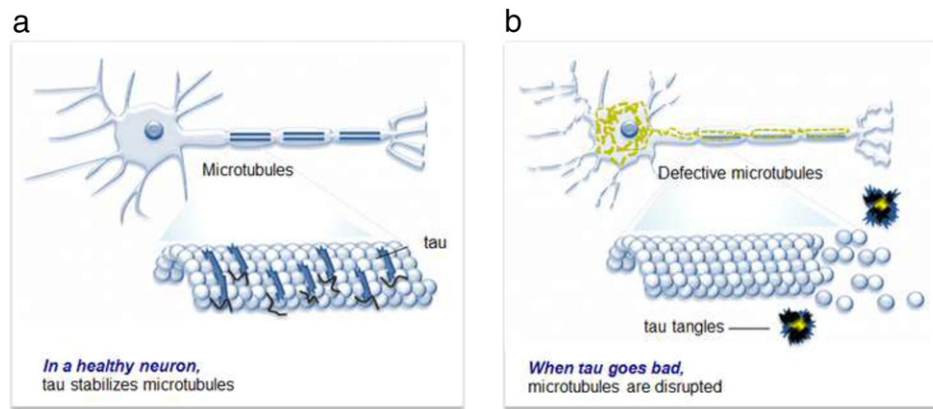


Fig. 1. Tau aggregation and neuronal degeneration [18]. (a) In a healthy neuron, tau stabilizes microtubules promoting axonal outgrowth and synaptic vesicle transport. (b) When tau goes bad, tau becomes neurotoxic aggregates and microtubules become dissociates.

cells in a number of ways [11]. First, aggregation of tau causes neuronal toxicity by reducing normal function of tau promoting microtubule stability. Also, the large filamentary tangles might be toxic to neurons by acting as physical barriers in the cytoplasm. Therefore, neurons containing tau tangles actively activate diverse cell metabolisms to get rid of the abnormal protein aggregates from cytoplasm [14,15]. This might be a great burden to a neuron that results in neuronal toxicity and neurodegeneration.

More recent studies suggest that, instead of the large insoluble filaments, soluble tau aggregates might play more critical roles in the onset and progression of disease prior to the development of NFT-induced neurotoxicity [6]. Especially hyperphosphorylated tau before NFT formation leads to microtubule disassembly, impairment of axonal transport, and organelle dysfunctions in neurons, leading to the neuronal cell apoptosis [3]. Also, the oligomeric species of tau may act as seeds for the aggregation of native tau, thereby promoting neurotoxic tau aggregation [8,16]. Accumulating evidence has suggested that tau aggregates are transmittable in neurons propagating as a prion-like manner [7,17].

Due to the pathological significance, tau becomes an important therapeutic target. Preventing tau aggregation becomes a potential strategy to cure neurodegenerative disorders associated with tau. So far, great effort has been made to identify molecular mechanism of tau aggregation and to reverse the processes. However, progress has been slow due to the lack of understanding the tau aggregation mechanism. Development of a reliable model for tau aggregation would be beneficial not only for identifying new therapeutic biomarkers but also for screening and evaluating drug candidates. Toward that, diverse tau aggregation methods have been developed: *in vitro*, cell-based, and *in vivo* models. Among the diverse models here we will review the *in vitro* and cell-based models for tau aggregation. *In vitro* tau aggregation methods have long been used for elucidating structural assembly of tau in the formation of PHFs. Cell-based models have recently developed to investigate the intracellular tau aggregation mechanism.

2. Multi-step Processes of Tau Aggregation

Contradictory to its pathological aggregation, tau is a naturally 'unfolded' protein, which is highly soluble in physiological condition [8,9]. To be a susceptible intermediate for aggregation tau molecules undergo a series of post-translational modifications and conformational changes in a neuron [19]. It is generally believed that tau aggregation is initiated by hyperphosphorylation (Fig. 2). Microtubule binding domains of tau contain a number of lysine residues, of which positive charges drive tau to bind negatively charged microtubules [20]. When tau is abnormally hyperphosphorylated, the balance is disrupted and tau dissociates from microtubules. Then, unbound tau undergoes conformational change to form a compact structure, called "Alz50 state" [21]. In this state, tau begins to aggregate and the further fibrillization is facilitated with proteolytic cleavages [1,13,22]. The truncated tau, named tau-66, assembles much faster than its native form [23]. NFTs are predominantly composed of paired helical filaments which appear to be made up of 10-nm filaments helically twisted with each other [24].

2.1. Tau Aggregation Assays In Vitro

To identify the ultra-molecular structure of PHFs, it is of obvious interest to reconstitute tau assembly process *in vitro* [12–14]. However, recombinant tau, which is purified from *Escherichia coli*, shows very little intrinsic tendency to aggregate *in vitro* due to the lack of a series of post-translational modifications required for aggregation. Over the last thirty years, the slow aggregation rate of purified tau has been improved by a combination of diverse advances (Table 1). First, recombinant truncated tau isoforms (e.g., the repeat domain alone aggregates) are more frequently used for *in vitro* tau aggregation, instead of full-length tau. Tau-repeat domains such as K18 or K19 alone aggregate much faster than the full-length tau (Fig. 3) [25,26]. Second, tau mutations such as P301L or Δ K280 occurring in FTDP-17 are known to enhance the β -sheet propensity to increase the aggregation reaction [27–31]. Third,

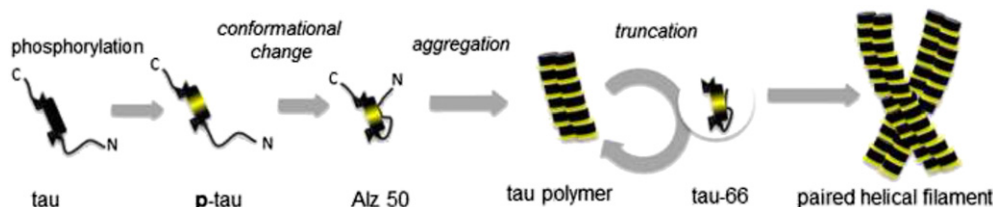


Fig. 2. Diagrammatic representation of tau aggregation [25].

Table 1
Tau aggregation assay *in vitro*.

Tau isoform	Mutants	Aggregation inducer	Incubation		Detection	Ref.
			Temp	Time		
Tau-40	–	Free fatty acid	37 °C	24–100 h	TEM	[38]
		Arachidonic acid	37 °C	3–25 h	ThS, TEM	[28,32]
		Heparin	30 °C	48–72 h	ThS, TEM	[33,34]
		Polyglutamate	30 °C	48–72 h	ThS	[34,37]
Tau-23	–	Arachidonic acid	37 °C	3–25 h	TEM	[26]
		Pre-aggregated PHFs	37 °C	20 h	ThS	[40]
		PHFs from AD patient	37 °C	20 h	ThS, TEM	[40]
K18	–	Heparin	37 °C	3–25 h	ThS, TEM	[26,34]
		Arachidonic acid	37 °C	3–25 h	ThS, TEM	[28,32]
		RNA	30 °C	–7 weeks	TEM	[41]
		Zinc	37 °C	1 h	ThS, TEM	[42]
K19	–	Heparin	37 °C	72 h	ThS, TEM	[43]
		RNA	30 °C	–7 weeks	TEM	[41]
K18	Δ K280	–	37 °C	2–4 days	ThS, TEM	[29,30]
K18	P301L	–	RT	7 h	LLS	[31]

addition of artificial cofactors also facilitate tau aggregation rate. Lysine-rich tau protein is extremely soluble in physiological conditions and it has very little intrinsic tendency to aggregate into filaments. So, the anionic cofactors screen the basic charges of tau and facilitate aggregation. There are two major classes of cofactors; polyanions (e.g., heparin, polyglutamate, or RNA) and fatty acids or fatty acid-like molecules (e.g., arachidonic acid, docosahexaenoic acid and alkyl sulfonate detergents) [26,28,32–34]. Poly-anionic cofactors such as heparin have a great efficiency in promoting the polymerization of tau fragments containing microtubule-binding domains (K18 or K19). Arachidonic acid also increases polymerization of full-length tau more rapidly than heparin.

These *in vitro* tau aggregation methods have been used in identifying structural assembly of tau in the formation of PHFs. Transmission electron microscopy technique (TEM) identifies that NFTs are predominantly composed of paired helical filaments [35]. Also, whether the fibers are paired helical filaments or not, PHFs isolated from Alzheimer's patients showed typical 80 nm crossover repeats. Whether it is artificial or not, these *in vitro* assays are commonly used to estimate the amount of tau aggregation in real time by using fluorescence probes such as thioflavin S (ThS); ThS is a widely used fluorescence probe to sensing the relative amount of β -sheet aggregates in solution [36,37].

3. Intracellular Modifications of Tau

Though the pathophysiological importance of NFTs in tauopathies, little progress has been made in understanding the packing of tau in the fibrils. The *in vitro* studies only provided the simple framework of tau aggregation hypothesis [38,44,45]. The cause and molecular mechanisms underlying tau aggregation remains still largely unknown. Progress has been slow because there is no reliable method for monitoring tau aggregation in physiological conditions, where tau is spontaneously altered and aggregated. Prior to or during NFT formation, tau undergoes numerous, and potentially harmful, modifications [39].

Among the diverse modifications of tau, phosphorylation induces the most critical change of tau leading to tau aggregation (Fig. 4) [46, 47]. Full-length tau protein has 80 serine and threonine residues and 5 tyrosine residues (Fig. 4b). In an intact cell, tau is constantly phosphorylated and dephosphorylated for the regulation of microtubule assembly. Once the balance is disrupted, tau is highly phosphorylated. The additional phosphates disrupt the charge balance between tau and microtubules, and tau dissociates from microtubules. Therefore, hyperphosphorylation is the critical event in the initiation of tau pathology [48]. Tau phosphorylation is tightly regulated by kinases or phosphatases [49,50]. Among diverse enzymes, GSK3 β is the most effective tau

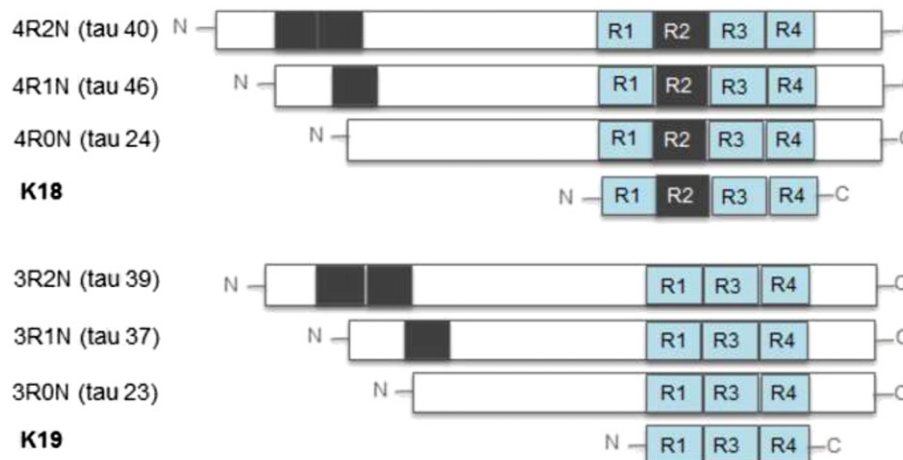


Fig. 3. Illustration represents human tau isoforms and truncated repeat domains. Human tau has six isoforms resulting from alternative splicing. The truncated tau repeat domains (K18 or K19) are known to facilitate tau aggregation.

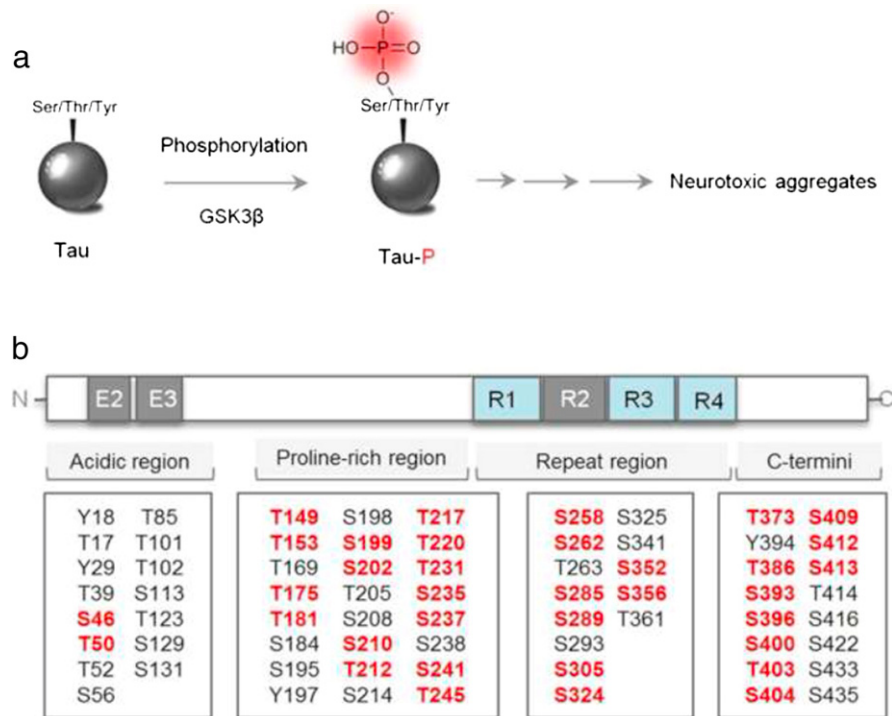


Fig. 4. Tau phosphorylation. (a) Initiation of tau phosphorylation by GSK3 β . (b) Putative tau phosphorylation residues. The red colored residues indicate GSK3 β mediated tau phosphorylation sites.

kinase in the human brain (Fig. 4a). Increased GSK3 β activity is directly linked to elevated levels of tau phosphorylation in AD patients [51–53].

Tau contains a number of lysine residues, of which positive charges are critical for binding to negatively charged microtubules. Similar to tau phosphorylation, acetylation of those lysine residues disrupts the charge balance between tau and microtubules resulting in tau aggregation. Lys280 is known as the major acetylation site and the increased acetylation promotes pathological tau aggregation [54]. Biochemical analysis of tau isolated from patient's brain also indicated the increased level of tau acetylation in AD [55]. Due to the diverse intracellular modifications of tau, tau aggregation has to be investigated in the complex cellular system. In the next section, we will introduce diverse cell-based approaches to induce and visualize tau aggregation in cells (Table 2).





3.1. Tau Aggregation Assays in Cells

As the simplest model of tau expression, Pouplana and co-workers expressed tau in *E. coli* and confirmed its aggregation in inclusion body by ThS stain [56]. Although it is an efficient way to enrich tau protein, bacterial system does not allow post-translational modifications required for spontaneous tau aggregation in cells. For the mammalian expression, Mandelkow's group generated tau-inducible cell lines [57, 58]. In their study, tau overexpression was toxic to the N2a neuroblastoma cells. Thus they expressed tau by using doxycycline-inducible system. The N2a cells expressing tau showed robust tau aggregation, which was detected by a fluorescence dye, ThS. Similarly, Jeff Kuret's group generated a tetracycline-insoluble tau cell line for tau aggregates. In their study, tau aggregation was promoted by the treatment of congo

Table 2
Induction of tau aggregation in cells.

Tau isoform	Mutants	Host cell	Expression	Aggregation inducer	Detection	Ref.
K18	Δ K280 Δ K280/PP (I277P/I308P)	N2a	Stable doxycyclin inducible	–	ThS antibody	[57]
Tau-40	–	HEK293	Stable tetracycline inducible	Congo red	Antibody	[59]
Tau-40	N279K Δ K280 P301L S305N V337M R406W	CHO	Stable expression	–	Antibody	[63]
Tau-40		QBI-293	Transient expression	Exogenous tau	Antibody	[60]
Tau-46		SH-SY5Y	Transient expression	Exogenous tau	Antibody	[62]
Tau-37					GFP	
Tau-40	P301L	NIH 3T3	Stable expression	–	GFP	[61]
Tau-39	V337M R406W				CFP YFP	

Table 3
Cell-based sensor for tau aggregation.

Tau isoform	Mutants	Tag	Host cell	Expression	Aggregation inducer	Detection	Ref.
Tau-40 Tau (1–421)	–		HEK293	Transient expression	GSK3 β	FRET	[65]
K18	Δ K280 P301L V337M I277P I308P		HEK293	Transient expression	K18	FRET	[66]
Tau-40 K18	Δ K280 I277P I308P		HEK293	Transient expression	GSK3 β	BiFC	[71]
Tau-40	–		HEK293	Stable expression	Forskolin okadaic acid	BiFC	[72]

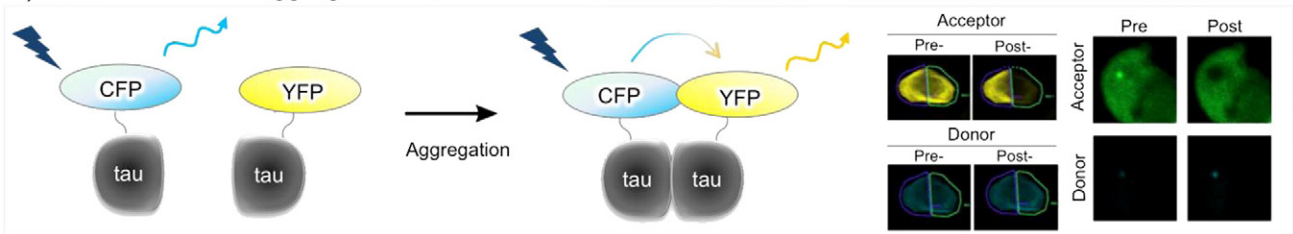
red, which is a known small-molecule agonist of tau aggregation, within cells expressing full length tau isoform [59]. In addition, Lee's group demonstrated intracellular tau aggregation that can be facilitated by the treatment of exogenous tau fibrils [60]. In their study, full-length of tau 40 was expressed in QBI-293 cells and tau aggregation was induced by the treatment of preformed tau fibrils, which act as a seed for intracellular tau aggregation. These studies successfully demonstrated that overexpressed tau could be aggregated in cells and also the aggregated tau induces cellular toxicity. However, these approaches require secondary methods to confirm intracellular tau aggregation such as or ThS or immune-stains against phosphorylated tau. To monitoring intracellular tau aggregation without any secondary detection methods, diverse fluorescence proteins (GFP, CFP, and YFP) are introduced to label tau and showed tau aggregation in living cells [61,62].

These cellular systems provide evidence of tau aggregation in cells and also enable the investigation of pathological mechanism of tau aggregation. However, these cell-based systems are not validated enough to quantify tau aggregation in living cells.

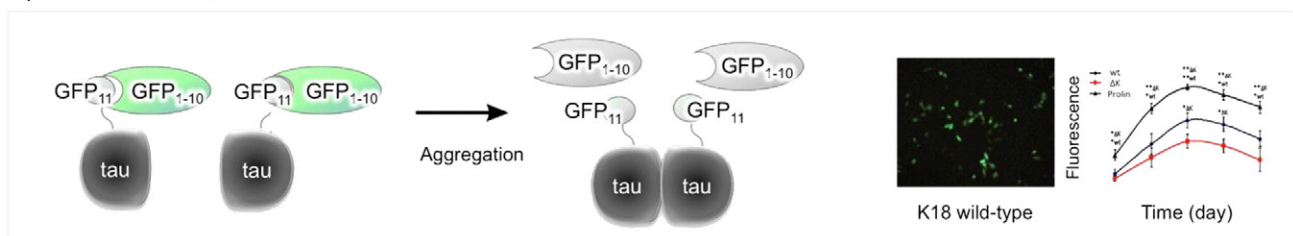
4. Cell-based Sensor for Tau Aggregation

A cell-based model that could monitor and quantify tau assembly in living cells would be a useful tool to investigate tau pathology and to discover methods to prevent and reverse the process. In this regards, diverse fluorescence protein technologies such as fluorescence resonance energy transfer (FRET) or bimolecular fluorescence complementation (BiFC) have been introduced to investigate tau–tau interaction in cells (Table 3).

a) FRET-based tau aggregation sensor



b) BiFC / GFP turn-off sensor



c) BiFC / YFP turn-on sensor

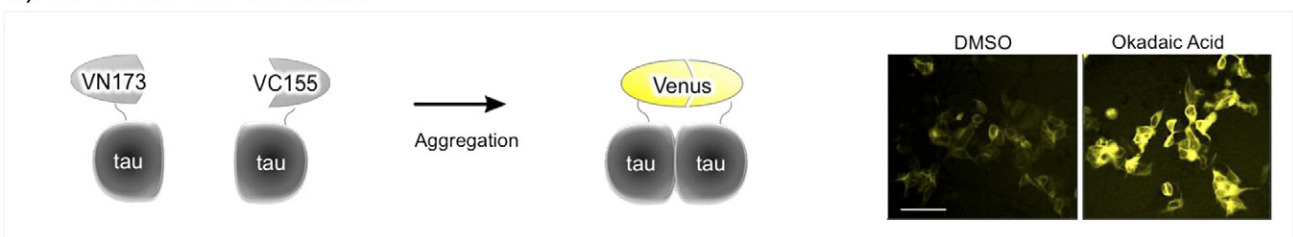


Fig. 5. Cell-based sensors for tau aggregation. (a) FRET-based sensor. Tau protein is fused to CFP or YFP. In the system, the FRET sensor is activated only when tau assembles. (b) BiFC/GFP turn-off sensor. Tau is fused to a smaller fragment of GFP (GFP11) and co-expressed with a large GFP fragments (GFP1–10). When tau exists as a monomer, GFP1–10 freely binds to GFP11 giving the strong GFP fluorescence. When tau aggregates, GFP1–10 cannot access to GFP11, resulting in the decrease of GFP fluorescence intensity. (c) Tau is fused to non-fluorescent N- or C-terminal fragment of Venus fluorescence protein (VN173 or VC155). The Venus fluorescence turns on only when tau assembles together.

4.1. FRET-based Tau Aggregation Sensor

First, Johnson's group introduced FRET technique to investigate tau–tau interaction in living cells. Fluorescence resonance energy transfer (FRET) is a process by which energy is transferred from a donor fluorophore (CFP) to an acceptor fluorophore (YFP) (Fig. 5a). The CFP and YFP are tagged to the proteins of interest [64]. Energy transfer between CFP and YFP occurs only when those proteins of interest are close enough (typically 2–6 nm). In their study, full-length tau and caspase-cleaved tau were labeled respectively with CFP and YFP, and then co-expressed in HEK293 cells [65]. When tau aggregation was induced by GSK3 β , the two different tau isoforms bind to each other resulting in the energy transfer between CFP and YFP. By measuring the FRET intensity, the level of tau aggregation could be quantified in a living cell. More interestingly, Diamond's group used this FRET technique to identify trans-cellular propagation of tau aggregation [66]. In their study various tau mutants (K18) was labeled with YFP or CFP, and then expressed separately. Then, cell-medium was collected from donor cells expressing tau–CFP and treated to adopter cells expressing tau–YFP. FRET microscopy showed that tau fibrils secreted into extracellular space could be taken up by other cells and aggregate with intracellular tau. FRET technology has great advantage in differentiating the aggregated tau species from non-aggregated tau in living cells.

4.2. BiFC: Turn-off Sensor

It is clear that FRET is one of the outstanding methods that can quantify protein–protein interactions in living cells. However, one drawback of the approach is that the use of quite huge fluorescence protein tagging that might interfere with the interaction between the proteins of interest. Therefore, there have been efforts to minimize the size of fluorescence protein tagging. The best example is bimolecular fluorescence complementation (BiFC) technique. To reduce the size of tagging, a fluorescence protein is split into two non-fluorescent fragments in the BiFC approach. Then the non-fluorescent constituents are tagged to the proteins of interest. Fluorescent turns on only when those proteins of interest are associated together [67–69].

Johnson's group applied this BiFC technique to visualize tau aggregation in living cells. A split green fluorescent protein (GFP) complementation technique was used to quantify tau aggregation *in situ* [70,71] (Fig. 5b). In this assay, full-length tau protein was directly fused to a smaller GFP fragment (GFP₁₁), and co-expressed in cells with a larger GFP fragment (GFP_{1–10}). When tau exists as a monomer or low degree aggregate, the complementary large GFP fragment is able to access the small GFP fragment fused to tau, leading to the association of the fluorescently active GFP. As a fluorescence “turn-off” approach, when tau aggregates, the reconstitution of active GFP is prohibited and then, intensity of GFP fluorescence decreases in cells.

4.3. BiFC: Turn-on Sensor

As a method of quantifying tau aggregation in living cells, the split-GFP complementation assay has been highlighted. However, as a fluorescence “turn-off” sensor, the split-GFP technique has an intrinsic limitation to monitoring the initial tau aggregation processes such as soluble tau intermediates. This limitation was overcome by using a Venus-based BiFC system. In this approach, Venus fluorescence protein is split into two non-fluorescent N- and C-terminal fragments (VN173 and VC155) and used to label tau [72] (Fig. 5c). As a fluorescence “turn-on” approach, Venus fluorescence turns on only when tau assembles together. There is little fluorescence background in basal condition, suggesting that most tau molecules exist as a monomer. Tau–BiFC fluorescence turns on dramatically when tau aggregation was stimulated by the treatment of small molecules inducing tau phosphorylation. As fluorescence “turn-on” technology, tau–BiFC approach enables to achieve spatial and temporal resolution of tau aggregation in living cells.

5. Concluding Remark

Due to the implications of tau pathology in diverse neurodegenerative disorders, tau becomes an important therapeutic target and great effort has been made to develop tau-targeted therapy. The primary therapeutic tactics considered include; (i) reduction of tau hyperphosphorylation using kinase inhibitors and phosphatase activators, (ii) activation of proteosomal degradation pathways of tau, (iii) tau clearance by immunotherapy, (iv) inhibition of tau aggregation using small molecules, and stabilizing microtubules. A reliable method to detect and monitor tau aggregation would accelerate understating tau aggregation mechanism and also expedite the development of tau-targeted therapeutics. Although innovative, the FRET-based tau aggregation sensor needs fine control to measure tau aggregation, thus it is not applicable for massive drug screening that needs robust screening platform. In contrast, the recently developed tau–BiFC “Turn-On” system allows simple and quantitative measurement of tau aggregation by measuring the increased YFP intensity in cells as an indication of tau aggregation. Also, as an established cell line, it allows 384-well based high-throughput drug screening providing reliable results. While the fluorescence protein tagging approaches enable monitoring intracellular tau aggregation, the use of a large tagging protein itself is a limitation of the approaches by adding an artificial manipulation of the system. We believe that this limitation can be overcome by developing a tau-selective fluorescence probe that can detect intracellular tau aggregation without the need for genetic manipulation. In this regards, the recently developed tau imaging probes give us a hope to facilitate the entire tau research [73,74].

Acknowledgments

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