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Direct visualization and profiling of protein misfolding and aggregation in live cells

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Abstract

Over the past few years, research tools have been developed to monitor the multistep protein aggregation process in live cells, a process that has been associated with a growing number of human diseases. Herein, we describe recent advances in methods that can either survey the distribution of aggregation at the level of the cellular proteome using mass spectroscopy or discern the multistep aggregation process of specific proteins of interest via fluorescence signals. Future development and application of such technologies are expected to provide insights on mechanisms, diagnosis, and treatment of diseases rooted in protein aggregation.

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Protein aggregation, Mass spectrometry, Fluorescence microscopy, Biosensor.

Introduction

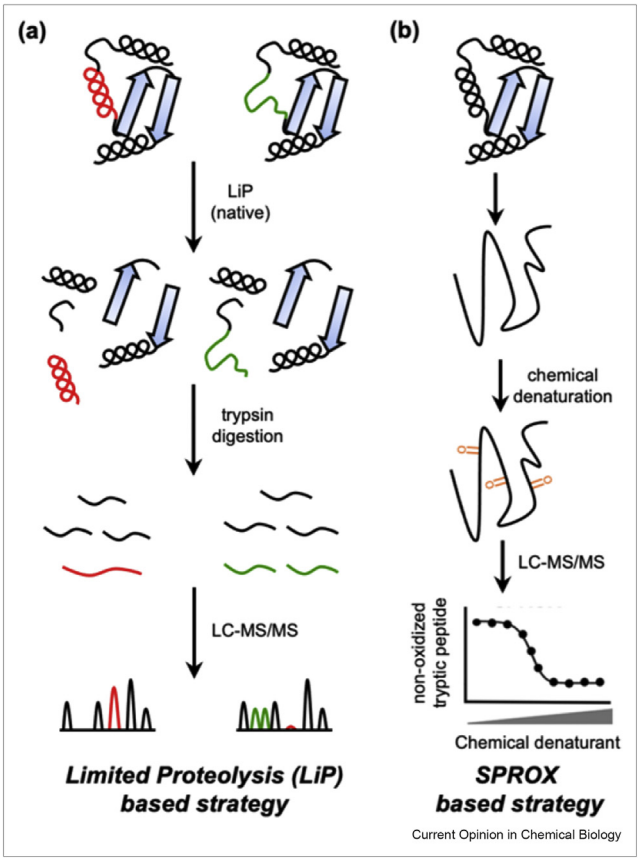
Protein misfolding and aggregation are implicated in the onset of numerous human diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease [1,2]. A common pathological hallmark of neurodegenerative diseases is the accumulation of insoluble protein aggregates in the central nervous system [3]. Therefore, monitoring protein aggregation in living cells is essential to understanding the molecular mechanisms of neurodegenerative diseases and to identifying drug candidates. It is generally recognized that protein aggregation is a multistep process: it initiates with a partial unfolding of the naturally folded protein, which further evolves into soluble oligomers and then insoluble aggregates [4]. Many environmental factors can perturb correctly folded conformation or inhibit protein folding, leading to protein misfolding and aggregation [5]. Once misfolded proteins are formed inside the cells, they can be either restored to their correctly folded state with the aid of chaperones or removed via degradation [6]. However, abnormal increases in the amount of misfolded proteins create a damaged or overloaded cellular proteostasis network, thus resulting in the accumulation of intracellular aggregates. To date, various methods have been developed to enable monitoring protein aggregation in cultured cells, including bacteria [7], yeast [8], and mammalian cells [9]. In this review, we summarize current strategies to probe protein aggregation in stressed proteome.

Mass spectrometry (MS)-based proteomics methods

MS is routinely used to profile protein abundance, post-translational modification, and protein interactions. However, it is believed that the environmental differences between the diluted solutions of purified proteins and the densely packed cellular environment could play an important role in protein conformation and stabilization [10]. MS-based techniques using a whole-cell approach have thus emerged to profile the native structural proteome within the complex cellular matrix.

In the limited proteolysis (LiP)–based strategy as an example (Figure 1a), cell lysate, where the proteins are in their native state, is firstly treated with a nonspecific protease at a low enzyme-to-substrate ratio and for a short time to generate large protein fragments. Then, complete trypsin digestion is conducted to different cleavage patterns, and the peptides are determined by bottom-up MS analysis [11]. This method is particularly useful in detecting protein structural changes. Feng et al. [11] used this approach to monitor over 1000 proteins simultaneously in yeast lysates and detected a structural change in about 300 proteins under different conditions, including fibril formation of α-synuclein, and unfolding of α-helix in myoglobin. Fitzgerald's group [12] combined LiP with stable isotope labeling with amino acids in cell culture

Figure 1



Workflow of limited proteolysis–mass spectrometry (LiP-MS) and stability of proteins from rates of oxidation (SPROX) to detect protein structural change. **(a)** LiP-MS. Proteomes extracted under different conditions are subjected to pulse proteolysis to generate structure-specific protein fragments. Then, they are denatured and fully digested to generate peptides amenable to bottom-up proteomics. Subsequently, all samples are subjected to mass-spectrometry analysis. **(b)** SPROX. The samples are treated with hydrogen peroxide so that the solvent-exposed thioether group is oxidized. Then, MS-based SILAC is used to quantify the extent of methionine oxidation as a function of the chemical denaturant.

(SILAC) to study the conformational changes of proteomes in breast cancer cell lines at different states and found several nucleic acid–binding proteins presented altered conformations in the different disease states. By applying cell lysates across a temperature gradient, Pascal et al. [13] used LiP for thermal profiling of roughly 6000 proteins, and the results revealed that thermal unfolding of a limited subset of essential proteins would lead to a cellular collapse.

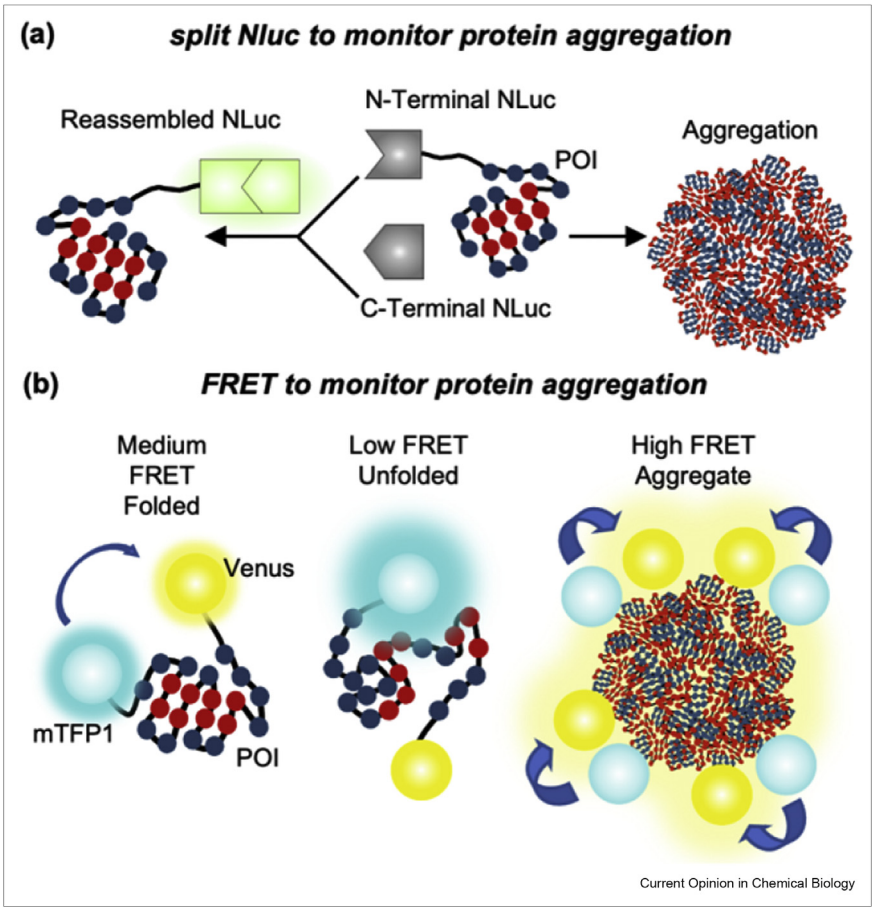
In stability of proteins from rates of oxidation (SPROX, Figure 1b), the chemical denaturant dependence of a hydrogen peroxide–mediated oxidation reaction involving methionine side chains is used to generate information about the global thermodynamic stability of proteins [14]. The close connection between protein folding stability and function was recently illustrated in several SPROX studies by Fitzgerald et al. [15,16]. SPROX was used to assay ~800–1000 proteins for changes in their protein folding behavior in MCF-10A, MCF-7, and MDA-MB-231 cell lines, associated with protein phosphorylation on the proteomic scale. The results of this work substantiate the close link between protein folding and function and could be further used for the study of disease processes.

A biosensor combined with fluorescent proteins or luciferases

Engineered metastable proteins flanked with two half-functional modules provide another solution to protein aggregate detection. Such modules can be split domains of a functional protein or fluorescence resonance energy transfer (FRET) pairs, both of which can only function when the two halves come into close proximity.

Split-protein reassembly, firstly observed by Fred Richards in 1958 [17], has established itself as a valuable tool to image and probe dynamic cellular processes. The two essential features of all split proteins are the lack of activity of each fragment in the absence of the other and the restoration of activity on fragment complementation or reconstitution [18]. Based on green fluorescent protein (GFP), a widely used genetically encoded fluorescent reporter, Cabantous et al. [19] successfully developed a self-assembling split GFP system, which is suitable for monitoring protein activity and interaction. However, this methodology is limited by the lag time of GFP chromophore formation after reassembly, potentially hindering real-time analysis. To solve this problem, Stain's group [20] proposed a new strategy for probing protein aggregation in living cells by using a self-assembling split luciferase, NanoLuc (NLuc), a 19.1 kDa luciferase enzyme that relies on the substrate furimazine to produce high intensity luminescence. The NLuc was split into two fragments: the N-terminal portion and C-terminal one (Figure 2a). *In vitro* experiments showed that both fragments were required for the production of a luminescence signal. The N-terminal NLuc fragment was fused to an aggregation-prone protein of interest (POI), and the C-terminal region was used to measure the level of aggregation of the POI. When the POI folded naturally, the addition of the C-terminal portion would produce a strong luminescence signal. Aggregation of the POI would bury the N-terminal segment inside and make it not available for reassembly with the C-terminal NLuc, leading to a weak signal. This assay has been used to monitor the aggregation of $A\beta_{1\text{-}42}$ [20], α-synuclein [21], amylin, and huntingtin [22] in living cells. Moreover, this strategy provides a platform to identify protein aggregation inhibitors [23].

Figure 2



Basic principles of the biosensor combined with fluorescent proteins or luciferases to detect protein aggregation. **(a)** Nluc is separated into two fragments: the N-terminal fragment and C-terminal one. The N-terminal is fused to POI. When POI is soluble, N and C move to the neighboring position, and the full-length reporter protein is reconstituted, resulting to a fluorescent readout by catalyzing the oxidative decarboxylation of furimazine. Once POI aggregates, the decreased amount of available N-terminus would lead to a decrease of emission intensity. **(b)** A POI is flanked with a FRET pair, mTFP1 as a donor and Venus as an acceptor. When the POI is at an unfolded or folded state, low or medium FRET signal can be detected, respectively. When the POI aggregates, the close distance between the donor and the acceptor results in a high FRET signal.

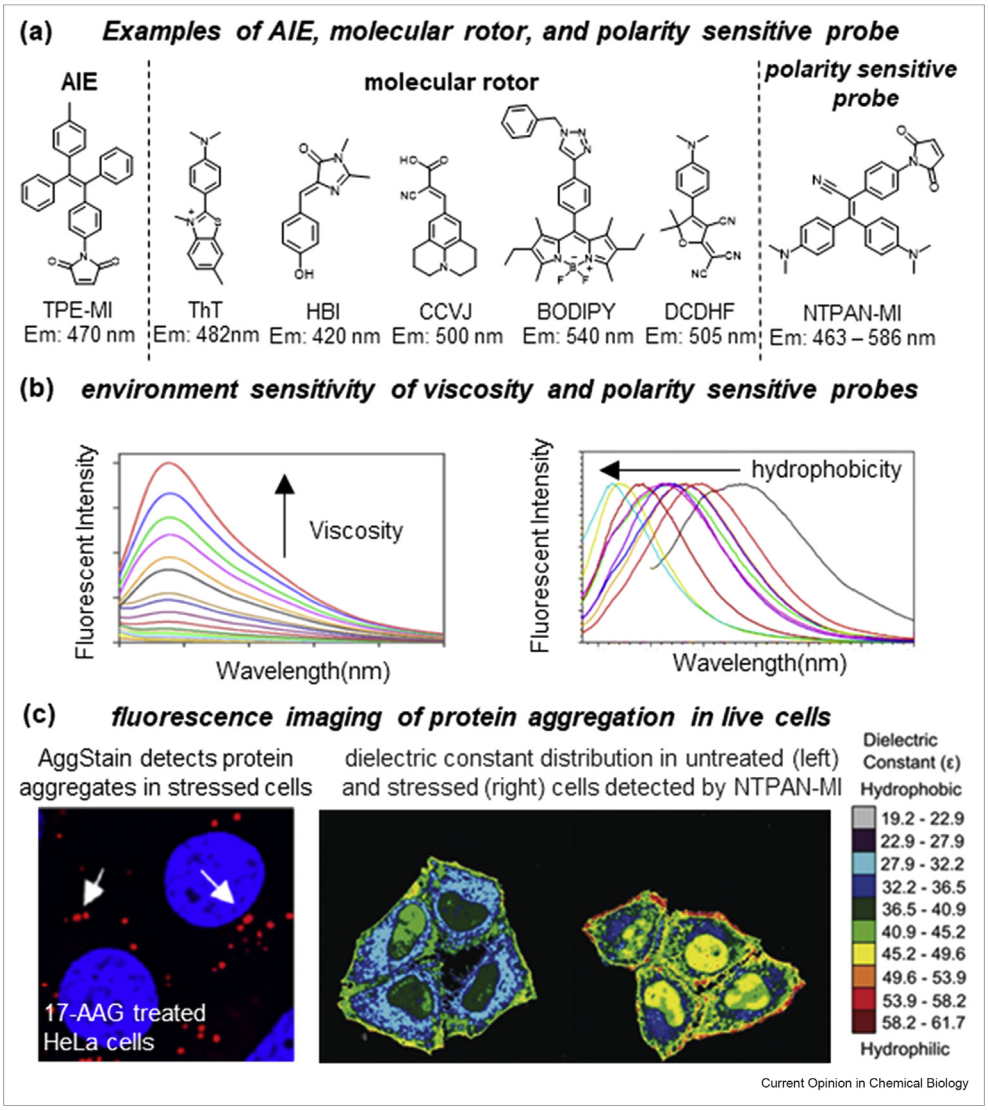
The FRET-based strategy involves a POI flanked with a FRET pair [24]. Gruebele et al. [25] imaged protein folding in live cells using phosphoglycerate kinase (PGK) that is fused with a FRET pair, AcGFP1 as a donor and mCherry as an acceptor. By microscopic imaging the green (donor) and red (acceptor) channels to derive the FRET signal, unfolding and refolding kinetics of PGK can be measured in live cells using a temperature jump setup. This method was further evolved into a platform to study the effect of low-molecular-weight solutes (<1 kDa) on the stability of PGK [26]. Following a similar strategy, Hatters et al. [27] flanked barnase with a FRET pair, mTFP1 as a donor and Venus as an acceptor, resulting in a biosensor to measure the proteostasis capacity using flow cytometry (Figure 2b). When barnase is in an unfolded or folded state, low or medium FRET signal can be detected, respectively. When barnase aggregates, the close distance between the donor and the acceptor results in a high FRET signal. The biosensor comprises a series of barnase mutants, which span a range of thermodynamic stability as quantified by ΔG_f values between −25 kJ/mol (most stable) and 1 kJ/mol (least stable). Rigorous quantification is achieved with this sensor, wherein flow cytometry is used to measure donor and FRET channels to derive the upper slope region corresponding to aggregated barnase and the lower slope region resulted from unaggregated barnase (a collection of folded and chaperone-bound unfolded proteins). Because these barnase mutants engage with the Hsp70 and Hsp90 chaperones, this sensor could

be used to quantify the activity of chaperones to alter the folding and aggregation of cellular proteins.

Environmental-sensitive fluorogenic small molecule probes

Fluorescence-based bioimaging has found particular interest because of its sensitivity, selectivity, high contrast, and versatility. However, for the always-on fluorophores, washing steps or time delays are needed to remove excess or unbound probes to generate sufficient signal-to-background ratios for imaging or sensing. Instead, a group of molecules can either turn on their fluorescence signal on viscosity increase or shift their emission wavelength in response to polarity changes in their surrounding microenvironment [28]. As protein aggregation involves changes in viscosity and polarity, it makes the fluorogenic molecules desirable probes for protein aggregation detection. The most frequently used fluorogenic probe for protein aggregation detection is thioflavin T (ThT, Figure 3a) [29]. On binding to amyloid fibrils, ThT gives a strong fluorescence signal because of the restriction of intramolecular rotation,

Figure 3



Fluorogenic small molecule probes for protein aggregation detection. **(a)** Chemical structures of ThT, TPE-MI, HBI, CCVJ, DCDHF, BODIPY, and NTPAN-MI. **(b)** Emission behavior of fluorogenic molecules. Rotor-based molecules show a turn-on fluorescent signal in a viscous environment because of the RIR, whereas the solvatochromic molecule shows a bathochromic shift in polar solvents. **(c)** Left: Fluorescence confocal images of the aggregated proteome in live HeLa cells induced by 17-AAG. Blue: nuclear stain and red: HBI derivative stained aggregated proteome. Right: Images of intracellular dielectric constant distribution in untreated and stressed cells stained by NTPAN-MI. This was adapted with permission from the study by Wan et al. and Owyong et al. [44,47]. RIR, restriction of intramolecular rotation.

whereas a negligible signal is detected for ThT alone [30]. However, it fails to recognize protein conformational changes, such as partially unfolding monomers and soluble oligomers, which are crucial to the formation of protein aggregates.

In recent years, molecular probes have been developed to detect these early events of protein aggregation. Aggregation-induced emission (AIE)—based molecules display no fluorescence on free intramolecular rotation in solution but exhibit strong fluorescent signals when their molecular conformations are restricted under rigid conditions [31]. In recent years, several groups have modulated AIE-based probes to detect the early events of protein aggregation [32—35]. As an example, Chen et al. [35] evaluated protein unfolding and proteostasis stress using tetraphenylethene-maleimide (TPE-MI, Figure 3a). TPE-MI remains nonemissive either in solution or aggregate state until the MI moiety is conjugated to thiol and the intramolecular rotation is inhibited in misfolded or aggregated proteins. When the protein is partially unfolded, the exposed free cystines and hydrophobic cores turn on the fluorescence of TPE-MI. Because of the requirements to switch on TPE-MI, it bypasses the false turn-on from accessible thiols on the folded protein surface and nonprotein thiols such as GSH, offering a better chance to monitor protein unfolding in live cells.

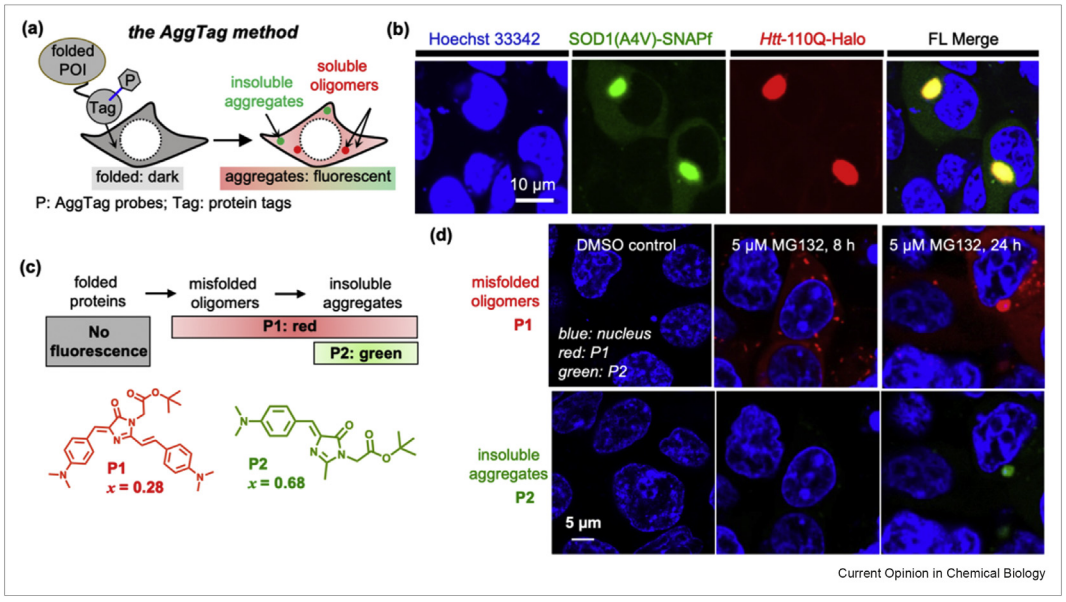
When proteins form misfolded oligomers and insoluble aggregates, molecular rotor—based fluorophores have also been applied to detect the increased viscosity of their microenvironment. A series of molecular rotor—based fluorophores have been used to detect protein aggregates, represented by CCVJ, the fluorescent protein chromophore core (HBI), and DCDHF (Figure 3a and b) [36—44]. Taking HBI as an example, these molecular rotors are nonfluorescent when synthesized outside their protein cavity because of rapid nonradiative decay via twisted intramolecular charge transfer (TICT). The fluorescence can be recovered by the inhibition of TICT in a viscous microenvironment. As a representation, Liu et al. [43] reported an HBI-based probe that is able to activate its fluorescence when binding to misfolded oligomers that are formed from purified superoxide dismutase (SOD1) or α-synuclein. Based on this scaffold, Wan et al. [44] reported the application of the HBI-based fluorogenic sensor, named AggStain, which can noncovalently and reversibly bind to proteome amorphous aggregates in stressed cells as demonstrated by the Hsp90 inhibitor 17-AAG—treated HeLa cells (Figure 3c). Meso-BODIPY is another type of molecule rotor whose potential energy barrier between the planar structure and the core-distorted structure could be overcome because of photoinduced electron transfer, leading to a nonradiative decay dominated energy relaxation (Figure 3a) [45]. The rotation between the two formations would be blocked when the molecule is embedded in a viscous environment. Nicolo et al. [46] reported a BODIPY derivative based on meso-BODIPY, whose fluorescent readout can be turned on by the soluble oligomers. Because of the early state detection of protein aggregation, they screened several inhibitors that can actively interfere with the fibril formation of $A\beta_{1-42}$.

In addition to viscosity, the microenvironment of misfolded proteins exhibits decreased polarity compared with folded proteins. Thus, polarity-sensitive probes have also been used in environmental-sensitive strategies for wash-free labeling (Figure 3b). A recent development of an AIE-based fluorophore exhibits strong solvatochromism by replacing one phenyl group with a cyano group (NTPAN-MI, Figure 3a), building up a push—pull system [47]. By analyzing the emission spectrum in different organelles, the newly designed system revealed that unfolded proteins in the ER are surrounded by a hydrophobic environment, whereas proteins in the nucleus are embedded in a relatively hydrophilic one. Once treated the cells with stress, the surroundings of ER proteins became even more hydrophilic because of the increased exposure of hydrophobic patches of proteins (Figure 3c).

The AggTag method to image the multistep aggregation pathway in live cells

To investigate the aggregation behavior of a specific protein in cells, Zhang et al. [43] have developed a method (named AggTag as aggregation tag) that combines self-labeling protein tags with fluorogenic molecules (Figure 4a). This method features small molecular AggTag probes, named AggFluor, that turn on fluorescence only on interaction with misfolded oligomers and insoluble aggregates because of the increased surface hydrophobicity and/or elevated intermolecular rigidity of these species compared with folded proteins. When AggFluor is covalently conjugated to a protein tag (HaloTag and SNAP-tag in our work) that is genetically fused to the folded POI, their fluorescence is quenched because the fluorophore is free to enter the TICT state. Formation of misfolded oligomers and/or insoluble aggregates of the POI, however, inhibits the intramolecular rotation, thus eliminates the quenching effect of TICT and yields turn-on fluorescence via intramolecular interactions with probes. Because HaloTag and SNAP-tag can be conjugated to any fluorophores that are installed with a reactive warhead, AggFluor can be technically any of the environmentally sensitive fluorogenic probes discussed in the previous section. Unlike conventional fluorescent protein fusion methods that track the location of late-stage aggregates via the appearance of fluorescent puncta, these probes specifically fluoresce in response to changes in protein

Figure 4



Basic principles of the AggTag method. **(a)** In cells expressing POI-Tag, stress conditions induce POI aggregation that forms misfolded oligomers and insoluble aggregates. **(b)** Confocal images of HEK293T cells co-expressing Htt-Q110·Halo and SOD1(A4V)·SNAPf. **(c)** Chemical structures of P1 and P2 and their corresponding viscosity sensitivity (*x*). **(d)** MG132-induced SOD1-A4 V-Halo aggregation at 8 and 24 h. At 8 h, P1 exhibited fluorescence in both diffusive and small granular misfolded oligomers, whereas P2 remained dark. At 24 h, P2 exhibited fluorescence only in perinuclear granular aggresomes and P1 remained to be fluorescent in both diffusive and small granular misfolded oligomers. This was adapted with permission from the study by Jung et al. and Wolstenholme et al. [42,48].

conformation and thus report on early events during protein aggregation.

The AggTag method can be used to simultaneously detect the aggregation of two proteins in the same live cells. As a demonstration, SOD1(A4V) and huntingtin exon 1 protein with 110 polyglutamine repeats (Htt-110Q), which tend to misfold and aggregate in cells on a variety of stress conditions, were fused to SNAP-tag and HaloTag, respectively, for orthogonal labeling (Figure 4b) [48]. Two AggFluor probes were conjugated to the POIs covalently, and *in vitro* study showed they were able to turn on their fluorescence when the POIs formed either soluble oligomers or insoluble aggregates. The orthogonal labeling allowed the visualization of both misfolded and aggregated form of huntingtin and SOD1(A4V) in a single live cell. This method enables multicolor imaging capacity which is suitable to address complex cellular events including regulation of protein aggregation and membraneless organelles. Furthermore, the viscosity sensitivity of the AggFluor probes could be finely tuned via incorporation of the electron density regulator into this chromophore [42] or installation of extended π-rich linkages between electron donors and acceptors [39]. As an example, P1 and P2 probes as shown in Figure 4c exhibit distinct viscosity sensitivity (as quantified by the *x* value), wherein P1 activates its fluorescence at a much lower viscosity than what P2 requires to activate its fluorescence emission. The difference in viscosity sensitivity and distinct emission spectra of these chromophores allows for the differentiation of misfolded oligomers from insoluble aggregates in live cells (as demonstrated by SOD1-A4V in the proteasome inhibitor MG132-treated HEK293T cells, Figure 4d). The simultaneous visualization of red signal from low-viscosity misfolded oligomers and green signal from high-viscosity insoluble aggregates shows a multi-step process of protein aggregation. In addition to the AggTag method, the same group also developed an aggregation-prone mutant of HaloTag, named as AgHalo to quantify cellular proteome stress and recovery [49]. A fluorogenic molecule was conjugated to the engineered AgHalo covalently, and *in vitro* study showed it was able to turn on the fluorescence when AgHalo formed either soluble oligomers or insoluble aggregates. By evaluating the fold-of-change of the fluorescent intensity, the extent of proteome stress could then be quantified. In a following study, additional AgHalo probes were developed to detect even milder proteome stress and to function at a wide range of wavelengths [41].

Perspective

Herein, we provide a concise review of multiple techniques that detect protein aggregation in cellular lysates or live cells. Understanding their advantages and limitations would better help researchers to select and

combine different approaches to solve specific scientific questions. Future challenges for the field would be how the folding and aggregation of either specific proteins or cellular proteome can be monitored in their native state, without the potential perturbation exerted by the genetically fused protein tags. Furthermore, RNA molecules with expansions of short nucleotide repeats have also been found to form aggregates both *in vitro* and in live cells [50]. It can be envisioned that proper methods would also be in demand to understand the process and property of RNA aggregates. In addition to research using cell culture models, suitable methods and compounds would be needed to image the multistep process of biological aggregates in animal models. We anticipate the existing and future methods would allow us to acquire a better understanding of the mechanism of the disease and eventually lead to drug discovery and therapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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