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Single-molecule microscopy for in-cell quantification of protein oligomeric stoichiometry

Huanhuan Chen, Xihong Xie and Tai-Yen Chen

Protein organization modification plays a vital role in initiating signaling pathways, transcriptional regulation, and cell apoptosis regulation. Simultaneous quantification of oligomeric state and cellular parameters in the same cell, even though challenging, is required to understand their correlation at the molecular level. Recent advances of fluorescence protein and single-molecule localization microscopy enables the determination of localizations and oligomeric states of target proteins in cells. We reviewed the fluorescence intensity-based, localization-based, and photophysical property-based approaches for in-cell quantification of protein oligomeric stoichiometry. We discussed their working principles, applications, advantages, and limitations. These results also imply the combination of methodologies targeting different biological parameters at the single-cell level is essential to uncover the structure-function relationship at the molecular level.

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Introduction

Modification of protein oligomeric stoichiometry (i.e. the assembly of proteins into multimers) serves as a critical regulator mechanism for multiple cellular processes [1–4], including G protein-coupled receptors related signaling [2], transcription regulation of the Wnt/β-catenin pathway [3], and voltage-dependent anion channel regulated apoptosis [4]. Recently, abnormal protein oligomeric stoichiometry, instead of large protein aggregate, has been considered as the critical pathway that leads to diseases [5–7]. By comparing neurons with specific stabilized SOD1 fibrils and with nonnative oligomers, Dokholyan *et al.* demonstrated that the nonnative trimeric SOD1-associated neurotoxicity serves a potential pathway for motor neuron impairment [6]. These exciting studies underline the importance of in-cell quantification of protein oligomeric state and indicate the need for a molecular understanding of the correlation between oligomeric state and these processes.

Various approaches have been developed to investigate the oligomeric stoichiometry. The *in vitro* methods, such as interferometric scattering mass spectrometry [8] and X-ray crystallography [9], study the oligomeric stoichiometry of purified proteins. These methods provide the best control of experimental conditions, but the cellular environment's effect is mostly missed. In contrast, fluorescence-based approaches such as cross-correlation spectroscopy [10,11], fluctuation spectroscopy [12–14], anisotropy intensity analysis [15], and complementation assay [16], solve this issue by probing dye-labeled proteins in cells. They provide valuable information on the average oligomeric stoichiometry of the target protein and its connection to a specific cellular function.

Despite the ensemble approaches confirm the connection, they only report the averaged stoichiometry of the target proteins. Information on relative populations between different stoichiometry and spatial distributions of different oligomers is averaged out and unobtainable. This knowledge gap limits the molecular understanding of the oligomeric stoichiometry-based regulatory mechanism and how unusual oligomeric status correlating with diseases. The recent advance of superresolution microscopy [17,18] approaches biophysical problems with a single-molecule perspective. Single-molecule localization microscopy (SMLM) follows target proteins one at a time in real-time, as well as the formation, interconversion, and dissolution of interaction intermediates [19–23]. Excellent spatial and temporal resolutions and probing in the native cellular environment make SMLM especially suitable for the in-cell determination of protein oligomeric state.

Here, we reviewed currently available single-molecule fluorescence-based methods to quantify protein oligomeric stoichiometry in cells, with a specific focus on the fluorescence intensity-based, protein localization-based, and fluorescence photophysical property-based approaches. We discussed their working principles, advantages, and limitations. We summarized the biophysical processes addressed by these approaches. Finally, we

highlighted other potential methods to understand the oligomeric stoichiometry-based regulatory mechanism.

Fluorescence intensity-based approaches

Fluorescence intensity has become one of the most popular and earliest adapted properties to investigate biophysical processes since Chalfie *et al.* [24] demonstrated the application of green fluorescent protein to study gene expression in 1994. By genetically introducing fluorophores into the target protein, the fluorescence intensity serves as an indicator of the abundance of the protein. The general experimental analysis starts with extracting individual proteins' fluorescence intensity within the region of interest (Figure 1a). Collecting the intensity of all spots gives the fluorescence intensity distribution (Figure 1b, left), and monitoring the intensity of each spots over time generates intensity trajectories (Figure 1c).

The fluorescence intensity-distribution approach works because the fluorescence intensity of a single fluorophore is characteristic under a given excitation. The existence of multiple fluorophores proportionally increases fluorescence intensity. Experimentally, the fluorescence intensity calibration curves are first determined by using fluorescent proteins with known stoichiometry (Figure 1b, middle). Fitting the intensity distribution of target proteins with the calibration curves (Figure 1b, right) informs the protein stoichiometry. This approach has been applied to multiple membrane-protein-related biophysical processes [25,26], including the activation of intracellular signaling pathways through antigen-induced oligomerization of immunoglobulin M containing B cell antigen receptor (mIgM-BCR) in healthy and diseased cells [27•]. In resting B cells, mIgM-BCRs exist predominantly as monomers and dimers (Figure 1d, left) but form higher oligomeric clusters upon stimulation (Figure 1d, right). In contrast, the diseased model (i.e. chronic lymphocytic leukemia (CLL)-derived BCR) prefers to oligomerize (Figure 1e, left) even under the resting condition, showing an opposite behavior compared to its healthy control (DG75 cells, Figure 1e, right).

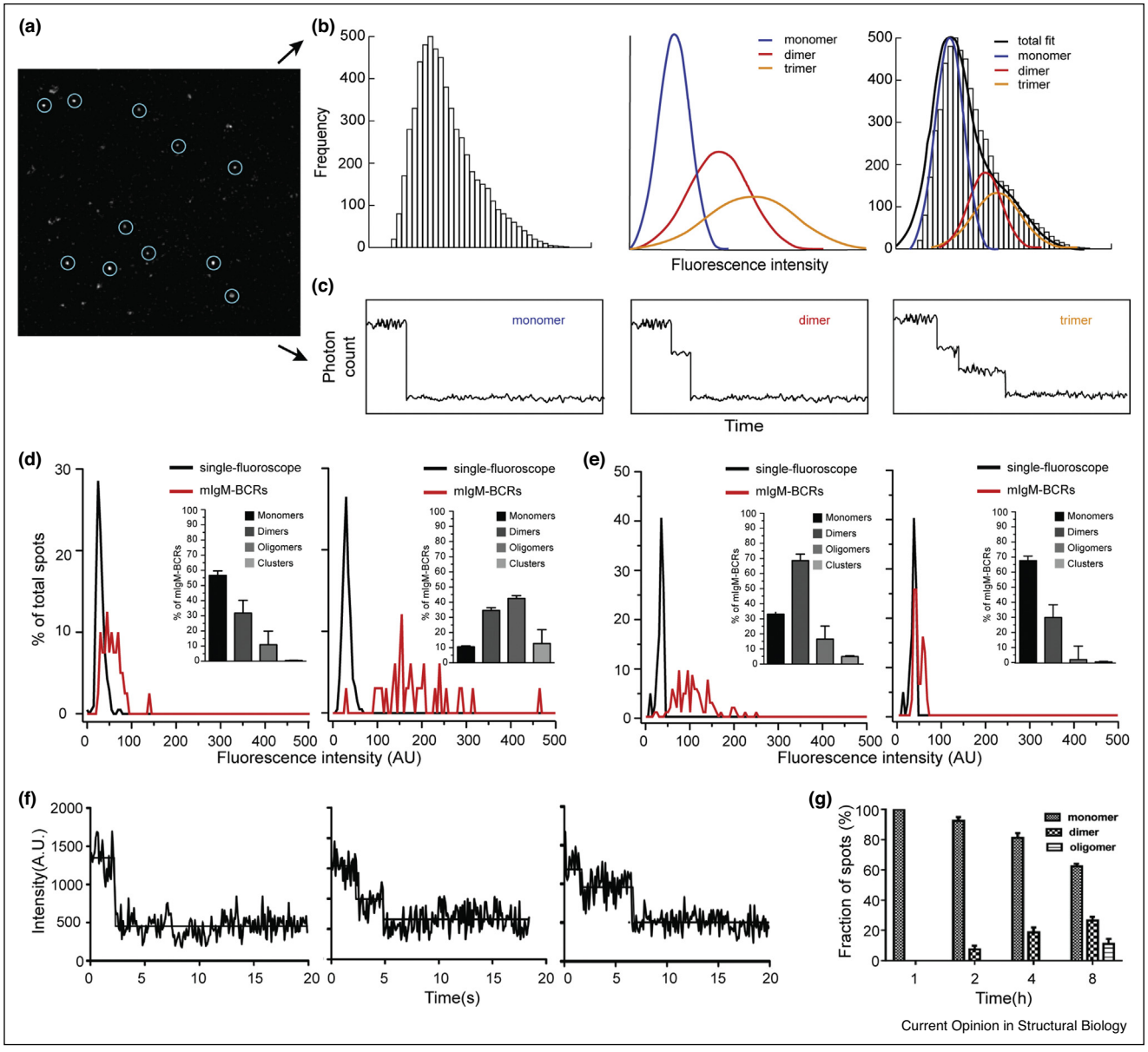
The photobleaching-step approach, another popular intensity-based approach, probes the stoichiometry by counting the photobleaching steps of single molecules' intensity trajectory. Under continuous laser excitation, photobleaching of individual fluorophore within one multimer results in an abrupt intensity decrease, where the number of photobleaching steps reports the subunit of the multimer (Figure 1c). The distribution of photobleaching steps of all multimers informs the relative populations of target protein existing in different oligomeric states. Note that the correction of pre-photobleaching before an actual measurement is needed to account for the undercounted steps and inaccurate stoichiometry [28•]. Photobleaching-step analysis [29] has been applied to study the regulatory mechanism of protein generation [30] and signal transduction function of G-protein-coupled receptors [31]. Song *et al.* [31] observed that chemokine receptor CCR3 exists as a mixture of monomer and dimer (Figure 1f) but forms oligomers with a higher concentration of CCR3 (Figure 1g).

Overall, fluorescence intensity-based approaches provide a simple way for quantifying protein oligomeric state. The extracted results are readily connected to the monomer number within one multimer and not affected by protein conformations. However, these approaches become less reliable when the oligomeric state goes beyond pentamer. With a higher oligomeric state, the intensity distribution profile gets broader, and the detection of discrete bleaching steps becomes difficult, both causing inaccurate analysis. Also, because of the choice of the constant fluorophores, fluorescence intensity-based approaches do not apply to proteins with a high cellular concentration, where the single-molecule imaging condition is no longer available. The invention of photoactivatable fluorescent protein [32] eventually resolved this dilemma and promoted the development of localization-based and photophysical property-based approaches.

Localization-based approaches

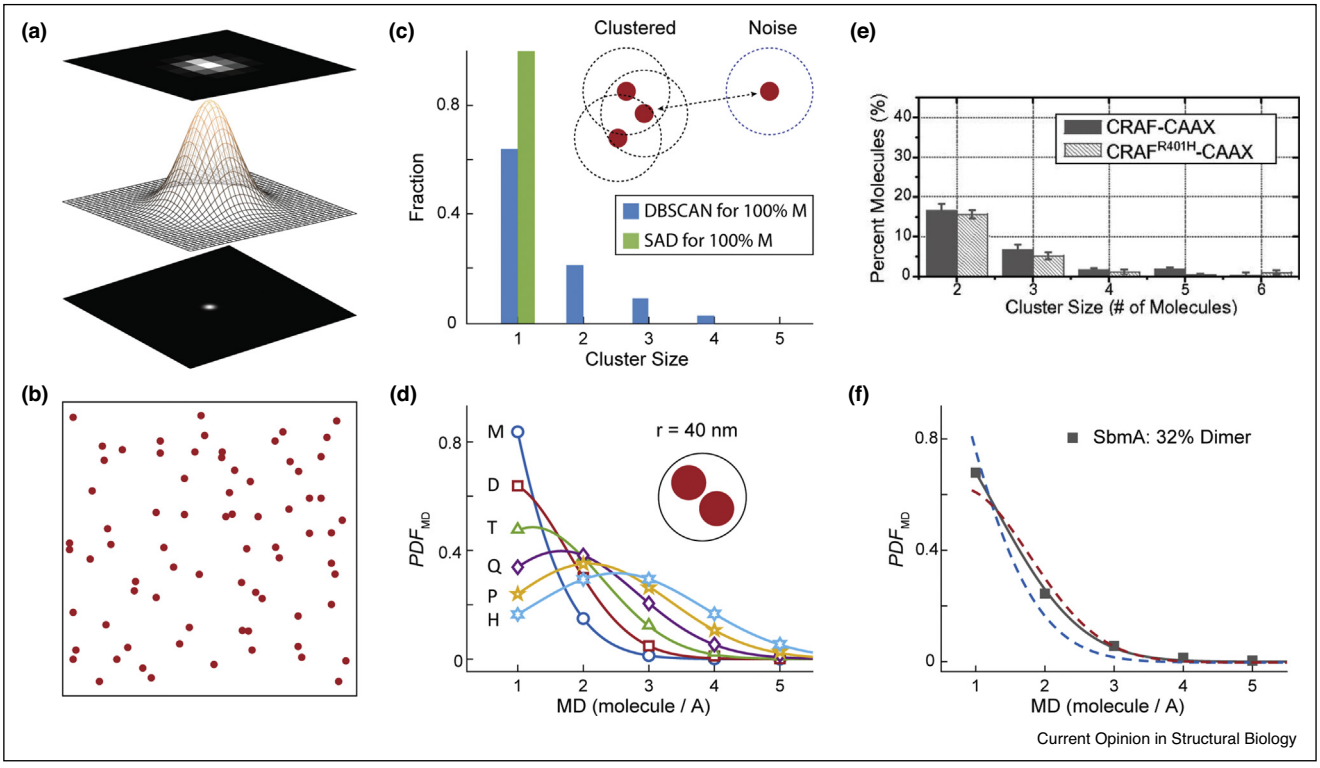
By fitting the point spread function (PSF), the diffraction-limited pattern from an infinitely small point source, with a Gaussian function (Figure 2a), single-molecule localization microscopy (SMLM) localizes target proteins with tens of nanometer spatial resolution. The superior spatial resolution of SMLM makes the localization-based clustering analysis another powerful approach for determining the oligomeric state. In principle, multimeric proteins would show up as a local cluster in the localization map (Figure 2b), where the cluster size informs their oligomeric stoichiometry. However, incomplete photoactivation [33] and photoblinking [34] of fluorophores cause a fractional and noisy localization map, which requires corrections in the subsequent analysis to pull out the underline stoichiometry of target proteins. Nan *et al.* [35] and Xie *et al.* [36••] addressed these issues by developing the simulation-aided density-based spatial clustering analysis with noise (SAD) and probability distribution function of molecule density (PDF_{MD}) algorithms, respectively. In both methods, the distribution of the number of proteins within a given area was first created from localizations obtained from SMLM and then fitted with a simulation set (Figure 2c and d). The simulation step not only allows directly integrated the photoactivation and photoblinking behaviors of fluorophores but also considers the effect of protein concentration. Nan *et al.* used SAD to study the dimerization and multimerization of the protein kinase CRAF, a component of a chain protein in the mitogen-activated protein kinases (MAPK) signaling pathway, under different cellular or therapeutic context (Figure 2e). Xie *et al.* adapted

Figure 1



Working principles and applications of fluorescence intensity-based approaches.
(a) Example of a typical single-molecule fluorescence micrograph. **(b)** Left: Intensity distribution is generated by histogramming the intensity of individual collected molecules. Middle: Fluorescence intensity calibration curves obtained from protein with known oligomeric stoichiometry. Right: The experimental intensity distribution of target protein is fitted by standard calibration curves to extract the relative populations of different oligomeric states. **(c)** Single-molecule intensity trajectories of monomer, dimer, and trimer show single-photobleaching, two-photobleaching, and three-photobleaching steps, respectively. **(d)** Fluorescence intensity distribution and relative populations among oligomeric states (inset) of mIgM-BCRs in resting Ramos cells (left) and cells with stimulation of anti-IgM F(ab')2 (right). Comparing to the single fluorophore intensity distribution (blacke curve), mIgM-BCRs (red curve) clearly exists in multiple oligomeric states. **(e)** Similar to (d) but for resting CLL-derived BCR (left) and its healthy control in DG75 cells (right). **(f)** Fluorescence intensity trajectories of monomeric (left) and dimeric (middle and right) CCR3 show one and two photobleaching steps, respectively. **(g)** Relative populations of CCR3 multimers with different expression levels. Longer expression time indicates a higher protein level. CCR3 forms oligomers at high protein concentration. Panel (d) and (e) reproduced with permission from Ref. [27•]. Copyright 2019 Springer Nature. Panel (f) and (g) reproduced with permission from Ref. [31]. Copyright 2018 American Chemical Society.

Figure 2



Working principles and applications of fluorescence localization-based approaches.
(a) Gaussian fitting of single-molecule point-spread function extracts the precise location of a molecule in SMLM. **(b)** Overlaying all extracted locations of molecules generates the localization map. **(c)** Clustering localizations of pure monomeric proteins by the density-based spatial clustering analysis with noise (DBSCAN) algorithm produces the distribution of cluster size (blue bar). Simulation-aided DBSCAN (SAD) employs simulation to extract the correct oligomeric stoichiometry (green bar). **(d)** In accordance with the experimentally determined cellular structures, PDF_{MD} simulates localization map assuming proteins are randomly distributed inside the cell. It groups spots within the 40 nm-radius area to generate the distribution of molecule density (PDF_{MD}). PDF_{MD} of a pure monomer (M), dimer (D), trimer (T), tetramer (Q), pentamer (P), and hexamer (H) show distinct distributions, which is useful to extract the average oligomeric state of target proteins. **(e)** SAD analysis reveals the CAAX motif fused CRAF as well as its R401H mutant form dimer and multimers on the cell membrane. **(f)** Fitting the distribution of molecular density of SbmA protein with simulation sets in (d) uncovers the 32% of SbmA existing in the dimer form. Panel (c) and (e) reproduced with permission from Ref. [35]. Copyright 2013 National Academy of Sciences. Panel (d) and (f) reproduced with permission from Ref. [36••]. Copyright 2018 American Chemical Society.

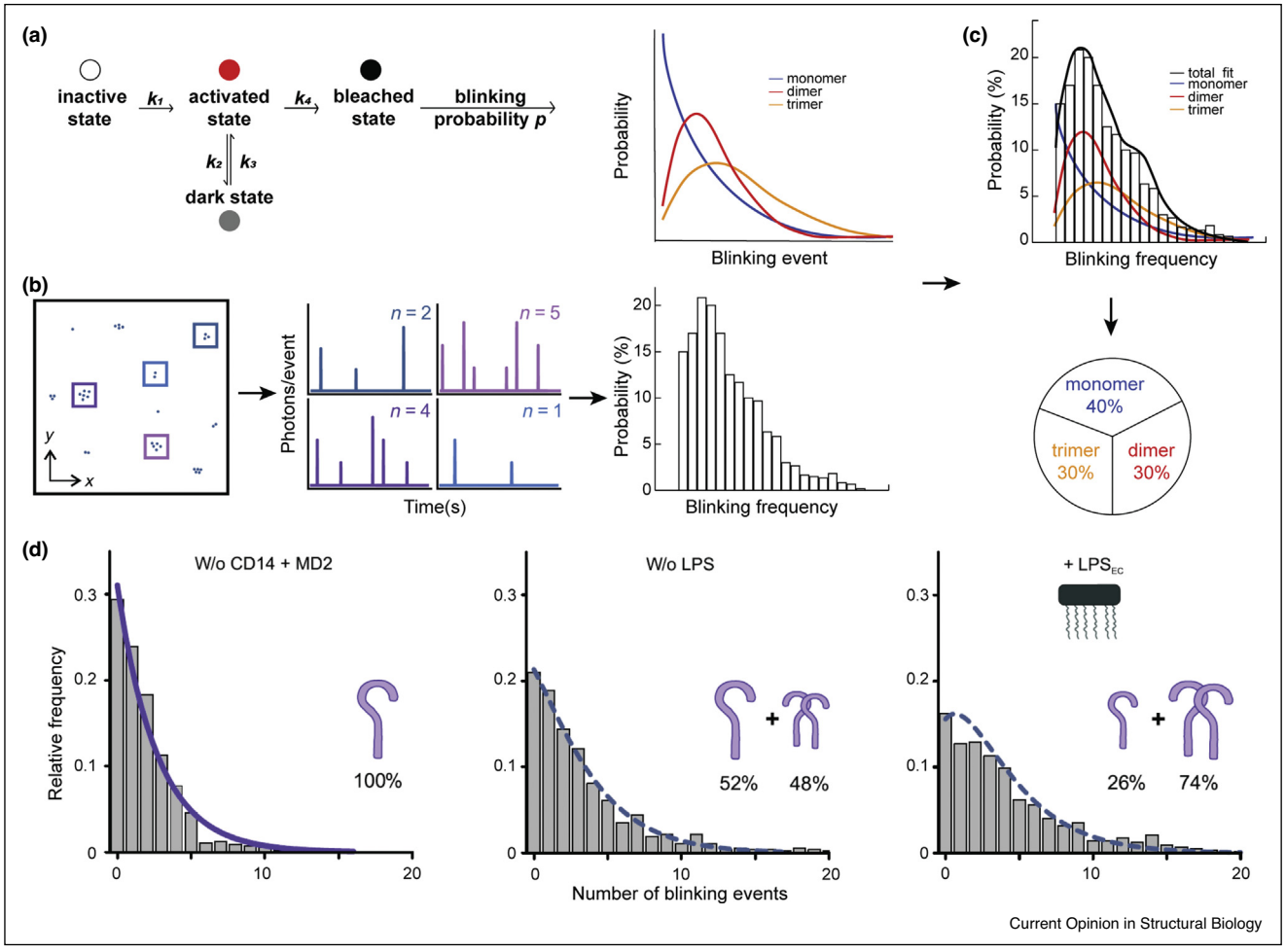
PDF_{MD} to show that membrane protein UhpT, a transporter for phosphorylated hexose molecules, exists as a pure monomer, and SbmA, a membrane protein uptakes drugs and peptide nucleic acid, is a mixture of ~30% of dimer and ~70% of monomer in the cell (Figure 2f).

The localization-based approach allows precise protein stoichiometric analysis and protein localization inside an intact cell, offering a unique perspective to uncover details of cellular processes at the molecular level. The SAD approach considers the noise level of SMLM images and accurately estimates oligomeric states under low and medium molecular densities. PDF_{MD} is especially suitable under the overexpression system. However, simulations in both approaches assuming a random distribution of proteins and can be further improved to handle the condition where proteins show heterogeneous cellular distributions.

Photophysical property-based approach

The photoblinking frequency of the fluorophore, even though not sensitive to the target protein's composition, has been used to quantify the oligomeric stoichiometry of the protein it attached. This approach typically uses photoactivatable or photoconvertible fluorescent proteins such as mEos variants and Maple 3 [37], whose blinking behaviors have been thoroughly investigated and described by a four-state kinetic model (Figure 3a, left) [38]. The four-state kinetic simulation suggests that a single fluorophore's photoblinking frequency shows a characteristic distribution [39]. The co-existence of multiple fluorophores in one multimer, each sharing identical

Figure 3



Working principle and applications of the photophysical property-based approach.
(a) Four-state kinetic model of photoactivatable fluorescent protein (PA-FP, left) and simulated calibration curve for distinct multimers (right). After activation, PA-FPs (red circle) can either enter the dark state (grey circle) reversibly or enter the bleached state (black circle). **(b)** Schematic generation of experimental blinking distribution. The intensity of each single spots will be monitored (left) over time to generate intensity trajectories (middle). The collection of blinking events from each trajectory gives the experimental distribution (right). **(c)** The experimentally determined distribution of blinking events is fitted to extract the average oligomeric stoichiometry of target protein. **(d)** Distribution of fluorescence blinking events of TLR4 protein under various co-receptor (i.e. CD14 and MD2) and agonistic and antagonistic treatments. Left: TLR4 protein dominantly exists in the monomeric form in the absence of CD14 and MD2. Middle: in presence of CD14 and MD2, TLR4 partially dimerized (monomer: 52%, dimer: 48%). Right: stimulation of agonist further increases the dimer to monomer ratio (monomer: 26%, dimer: 74%). Panel (b) and (d) reproduced with permission from Ref. [43]. Copyright 2017 The American Association for the Advancement of Science.

blinking kinetics, results in a blinking frequency distribution change. The blinking frequency distributions of proteins with specific stoichiometry typically are obtained by the stoichiometric controls (i.e. pure monomer and dimer) (Figure 3a, right) and serve as the calibration curves. These distributions are then compared to that of the target protein for stoichiometry determination. The experimental blinking-event distribution of the target protein is constructed from the fluorescence intensity trajectories of individual molecules (Figure 3b). The experimentally determined distribution is fitted with the calibration curves to extract the average oligomeric stoichiometry and the relative populations among different oligomeric states (Figure 3c).

The photoblinking approach is widely adopted in the field [40,41] since its invention [42] — for example, Krüger *et al.* [43] study the oligomeric state of the pathogen recognition receptor Toll-like receptor 4 (TLR4) and its correlation with signal transduction events in live cells. In the presence of its co-receptors cluster of differentiation 14 (CD14) and myeloid differentiation protein 2 (MD2), TLR4 shifts its oligomeric state from pure monomeric (Figure 3d, left) to a mixture of monomeric and

dimeric forms (Figure 3d, middle). Agonistic and antagonistic treatments drive TLR4 to different oligomeric compositions (Figure 3d, right). These findings demonstrate the ligand-dependent dimerization of TLR4 in the cellular environment and shed light on the molecular understanding of biased signaling. Baldering *et al.* [44••] further advanced this approach by including detection efficiency in their statistical model. This improvement considers that only fractional fluorophores are detectable during the imaging process, which is especially essential for the high-order oligomers.

Overall, the photoblinking approach is a well-suited methodology for protein oligomeric stoichiometry quantification. It accurately distinguishes different multimers by comparing the blinking-event distribution between experimental results and the reference. So far, photoblinking analysis applications are limited to membrane proteins even though application to cytosolic proteins should be feasible.

Concluding remarks

Single-molecule fluorescence microscopy offers multiple approaches to quantify the protein oligomeric states in cells. Besides the approaches above, several techniques may also play important roles in quantifying protein oligomeric states. For example, both single-molecule Forster resonance energy transfer (smFRET) [45–47] and interferometric scattering mass spectrometry [8,48] have demonstrated direct and accurate recognitions of bio-complex *in vitro* at a single-molecule level. Fluorescence anisotropy intensity (FAI) [15,49,50•] also distinguishes multimers at the ensemble level. Advancing smFRET to *in-cell* detection or performing FAI at the single-molecule level, on top of multimer determination, will further allow quantifying interaction dynamics. Finally, the combination of methodologies targeting different biological parameters at the same cell provides an ideal platform to uncover the structure-function relationship at the molecular level.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Huanhuan Chen: Conceptualization, Writing - original draft, Writing - review & editing. **Xihong Xie:** Conceptualization, Writing - original draft, Writing - review & editing. **Tai-Yen Chen:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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- •• of outstanding interest

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