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Feeling the vibes: Vibrational spectroscopic imaging of biomolecular assemblies in their natural environment **FREE**

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ABSTRACT

Biomolecular assemblies form via intramolecular interactions and serve important biological functions. The most characterized biomolecular assemblies are amyloid fibrils, which are associated with neurodegenerative diseases. Advances in microscopy techniques enabled characterization of the morphology of these assemblies, but so far, failed in detailed structural characterizations. Vibrational spectroscopic imaging presents unique advantages to studying biomolecular assemblies in their natural environment due to the sensitivity of vibrational spectra to protein structural changes, especially β -sheet enrichment in amyloid fibrils. High-resolution hyperspectral images originating from distinct vibrations of chemical bonds provide label-free characterizations of biomolecules, including proteins, lipids, and nucleic acids. In this review, we first briefly introduce infrared and Raman-based spectroscopy and their biological interpretation. We then review applications adopting Fourier transform infrared-based, mid-infrared photothermal-based, and Raman-based approaches in tissue and cells, especially live cells. Finally, we discuss how these technologies are evolving to study biomolecular assemblies beyond amyloid fibrils.

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characterized by a very rigid, thermodynamically stable structure.² They are generally composed of one or a few types of proteins that assemble in stacked β -sheet conformation, forming a “unidimensional crystal.”² Amyloid fibrils are particularly known for their relationship with neurodegenerative diseases.^{3,4} However, the formation of amyloid fibrils has also been involved in signaling,^{5,6} storage of peptide hormones,⁷ and as a main component of the extracellular matrix used by biofilm forming bacteria,⁸ in the form of curli fibrils.⁹

Among macromolecular assemblies, amyloids are only one extreme of a continuum of states carrying different material properties, such as liquid droplets and hydrogels.¹ Distinct strengths and types of intermolecular interaction determine the biophysical properties of the assembly, such that different types of assemblies can form separate biomolecular compartments, or membrane-less organelles.¹⁰ For this reason, the terminology recently shifted from defining specific types of assemblies to referring to this class of biomolecular phenomena as “condensates.” Moreover, different types of condensates can interconvert or evolve over time. For instance, the protein Fused In Sarcoma (FUS) forms very dynamic condensates that evolve into an amyloid fibril.¹¹ A similar phenomenon has been observed by us and others also in aggregates formed by the Huntington's Disease protein huntingtin,^{12–14} for the protein hnRNPA1,¹⁵ and finally in A β fibril formation.¹⁶

Structural and morphological characterization of biomolecular assemblies *in vitro* has culminated with the structural reconstruction at the subatomic level of fibrils of multiple disease-related proteins.¹⁷ However, the diversity of interactions, the transient nature of some of these macromolecular assemblies in cells, and their wide variety in sizes make structural characterization of biomolecular assemblies in their physiological state (i.e., the cell) a challenging task. Nevertheless, progress has been made in revealing structure and morphology of protein aggregates *in vitro* and in cells.¹⁸

Super-resolution techniques, such as stochastic optical reconstruction microscopy (STORM) and photo-activated polarized microscopy (PALM), have been used in the past decade for reconstructing the structure of amyloid fibrils at the nanometric resolution in cultured cells,¹⁸ as well as in human cryopreserved brain samples.¹⁹ Fluorescence-based super-resolution techniques, such as STORM and PALM, require labeling the amyloid fiber with photo-activatable dyes, generally performed using antibodies, irreversible chemical modification, or fusion of fluorescent proteins. Each of these methods carries its own disadvantages. Labeling with antibodies may introduce artifacts due to nonspecific binding; not all condensates can be labeled without perturbing their properties; and the samples need to be fixed. Fusion of fluorescent proteins guarantees homogeneous labeling within the condensates but could introduce artifacts due to the properties of the fluorescent protein itself,²⁰ such as tendency to oligomerize, or, the opposite, charge repulsion, or changes in solubility.^{21–23} Finally, chemical labeling has been explored as a solution due to its high brightness and a wider palette of wavelengths to deploy,¹⁸ but again the irreversible binding of the fluorophores could affect the mechanisms of condensation. In addition, chemical labeling is possible exclusively in fixed samples, or when the protein to study is chemically modified outside the cell and then subsequently delivered.²⁴

The advent of cryo-electron microscopy also revolutionized the study of amyloid fibrils. For instance, aggregates of A β , α -synuclein, and tau have been characterized in brain samples of deceased

patients.²⁵ This powerful technique enables almost-atomic resolution of the structure of amyloid fibrils in cells without the need for fluorescent labeling. Very recently, this detailed structural characterization has been extended to less rigid protein condensates, such as protein liquid droplets.²⁶ While this technique enables extremely powerful characterization of complex macromolecular assemblies, its high cost and high complexity hinder widespread use, and the samples need to be vitrified before being studied, which prevents live imaging.

Vibrational spectroscopic imaging expands the toolbox to study biomolecular assemblies in their natural environment. Vibrational spectroscopy can identify molecules based on their distinctive vibrations of chemical bonds, free of labels. Moreover, the advancement from spectroscopy to spectroscopic imaging has enabled broad applications in complex biological systems (Fig. 1).²⁷

Here, we first introduce two complementary vibrational spectroscopic approaches: infrared (IR) and Raman spectroscopy, focusing on their unique advantages for studying protein structures and their ability to resolve different macromolecules. Next, we review works studying biomolecular assemblies using vibrational spectroscopic imaging techniques, grouped by Fourier transform infrared (FTIR)-based, mid-infrared photothermal (MIP)-based, and Raman-based approaches. We briefly introduce the applications *in vitro* and then focus on studies in tissue and cells, especially live cells. Finally, we discuss how continuing technological advances could open new opportunities to biomolecular assemblies beyond amyloid fibrils.

VIBRATIONAL SPECTROSCOPY FOR BIOMOLECULAR ASSEMBLIES

Protein structure by vibrational spectroscopy

Vibrational spectroscopy includes two complementary techniques: infrared (IR) and Raman spectroscopy. Vibrational spectra encode protein structural information, and the high local protein density in biomolecular assemblies enables high signal-to-noise ratios, making vibrational spectroscopy techniques well positioned to study the structural properties of macromolecular assemblies. The amide I band, originating mainly from the C=O stretching in the peptide bonds of the protein backbone, is most sensitive to protein secondary structures, such as α -helix, β -sheet, and β -turn, in both IR and Raman spectroscopy [Fig. 2(a) and Table I].^{28–31,34} Moreover, the common characteristic of amyloid fibrils, cross- β fold,³⁵ can be spectroscopically differentiated from the native β -sheet, like those in green fluorescent protein (GFP). Amyloid fibrils present a distinctive amide I peak clustering between 1611 and 1630 cm^{-1} , while native β -sheets are between 1630 and 1643 cm^{-1} for purified proteins [Fig. 2(b)].³⁶ Another unique advantage of IR spectroscopy for protein aggregation lies in the differentiation between intermediate oligomers from mature fibrils: oligomers are associated with the anti-parallel β -sheet, with a band around 1695 cm^{-1} , which disappears when fibrils form. The intensity ratio I_{1695}/I_{1630} has been used to represent the percentage of anti-parallel vs parallel β -sheets, termed the “ β -sheet organizational index” [Fig. 2(c)].³⁷

Infrared and Raman spectroscopy for biological applications

Beyond protein structure, both IR and Raman spectroscopy can provide rich information regarding the interaction of lipids, nucleic

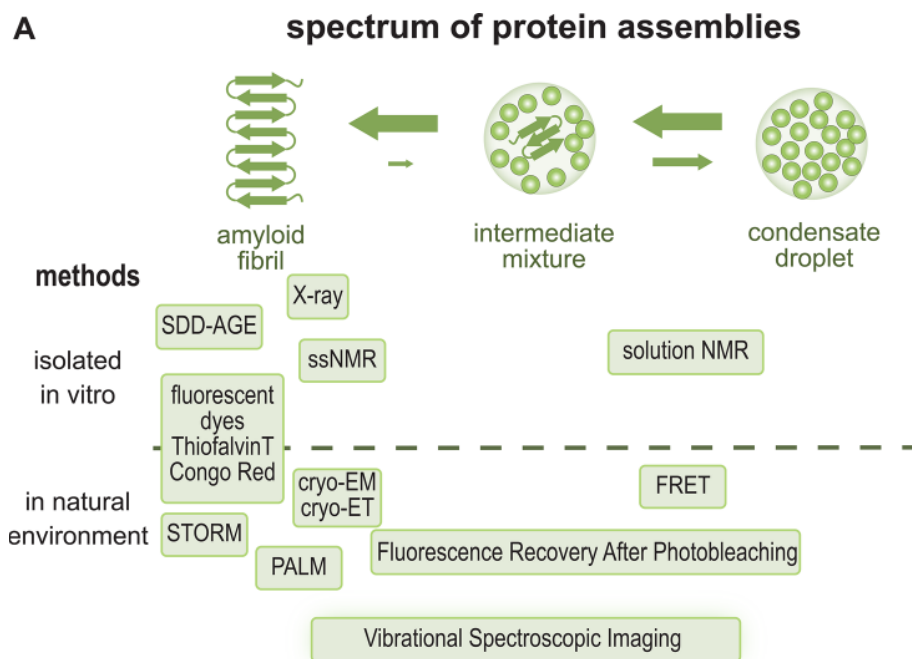
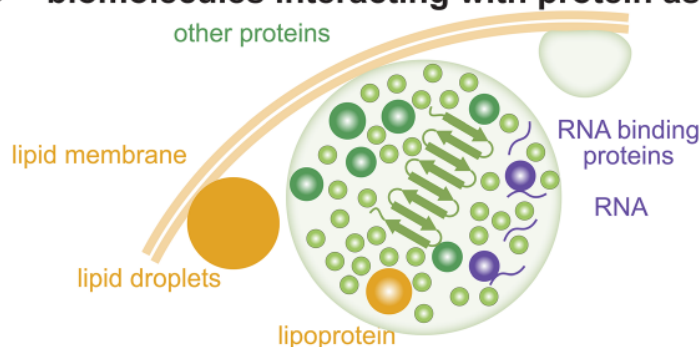


FIG 1 a) Overview of protein assemblies and methods to study them isolated *in vitro* or in their natural environment. b) Other biomolecules, including lipids and nucleic acids, can interact with proteins in protein assemblies.

B biomolecules interacting with protein assemblies



acids, or carbohydrates within biomolecular assemblies. Each of these biomolecules has distinct spectral features in both IR spectroscopy^{45,46} and Raman spectroscopy^{33,47} [Fig. 2(d)]. Additionally, there is a cell silent window ($1800\text{--}2700\text{ cm}^{-1}$), where endogenous biomolecules have no peak. This window has been exploited to introduce vibrational probes with biorthogonal nontoxic alkyne ($\text{C}\equiv\text{C}$), nitrile ($\text{C}\equiv\text{N}$), or azide ($\text{N}\text{--}\text{N}\text{--}\text{N}$) groups [Fig. 2(d)].^{48–51} These minimal chemical modifications introduce signals in the silent window that report on metabolic activities, such as trehalose trafficking⁵⁰ and enzyme activity.⁵¹ Additionally, isotopic labeling, which introduces heavier atoms like deuterium (D), ^{13}C , ^{15}N , and ^{18}O into the metabolites, introduces red-shifted peaks. By comparing the original peak and the red-shifted peak due to isotopic effects, metabolic activities could be measured, such as glucose metabolism,⁵² de novo protein synthesis,⁵³ and de novo lipid synthesis.⁵⁴ Therefore, vibrational spectroscopic imaging can expand the layers of information from morphological (visible light) to chemical

(endogenous IR absorption and Raman scattering) and finally to functional (vibrational probes and isotopic labeling).

BIOMOLECULAR ASSEMBLY BY FOURIER TRANSFORM INFRARED (FTIR)-BASED APPROACHES

FTIR spectroscopy and *in vitro* studies

Fourier transform infrared (FTIR) spectroscopy can collect high-resolution IR absorption spectrum data covering a wide spectral range and has been frequently used to study protein aggregation *in vitro*.⁵⁵ The use of FTIR to study purified proteins has paved many foundations in IR spectral analysis. For example, the differentiation between amyloid fibrils and native β -sheet was established by an FTIR study of a large number of purified proteins [Fig. 2(c)].³⁶ FTIR can also follow the longitudinal growth of fibrils and identify features of intermediate oligomers [Fig. 2(d)].³⁷

Vibrational Spectroscopy for Biomolecular Assemblies

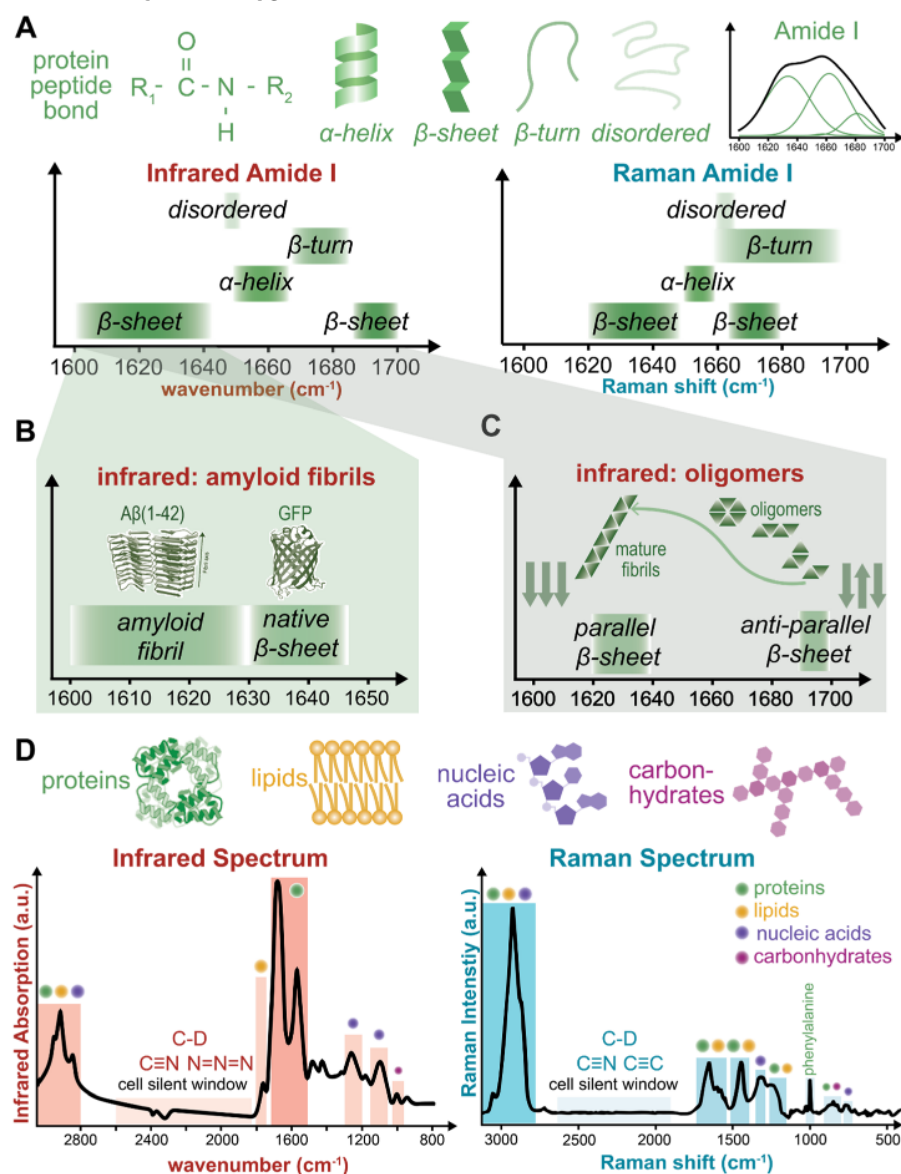


FIG 2 Resolving protein structures by vibrational spectroscopy. a) The amide I region is sensitive to protein secondary structure. Reproduced with permission from Barth, *Biochim. Biophys. Acta* **1767** (9), 1073–1101 (2007); Kong and Yu, *Acta Biochim. Biophys. Sin.* **39** (8), 549–559 (2007); Yang *et al.*, *Nat. Protoc.* **10** (3), 382–396 (2015); Maiti *et al.*, *J. Am. Chem. Soc.* **126** (8), 2399–2408 (2004). Copyright Authors, Licensed under Creative Commons Attribution CC BY license.^{28–31} b) IR spectroscopy can differentiate a cross- β fold in amyloid fibrils from the native β -sheet in GFP. c) IR spectroscopy can differentiate oligomers from mature fibrils. d) Typical IR and Raman spectra of biological materials. Left: Reproduced with permission from Al-Kelani and Buthelezi, *Skin Res. Technol.* **30** (6), e13733 (2024). Copyright 2024 Wiley.³² Right: Reproduced with permission from Xu *et al.*, *Cancers* **13** (7), 1718 (2021). Copyright 2021 MDPI.³³

Despite the broad applications of FTIR spectroscopy, developing it into imaging biological samples is challenging, limited by (1) long wavelength of infrared light leading to low spatial resolution (2–10 μm), (2) low brightness of conventional thermal IR sources leading to low sensitivity and slow speed, and (3) strong water absorption of aqueous biological samples.

Synchrotron-based IR microscope and in situ studies of brain tissues

The emergence of the synchrotron infrared source, which provides much greater brightness, enabled the development of

synchrotron-based IR microscopes and *in situ* study of brain tissues. One of the earliest uses was to examine the secondary structure of amyloid deposits containing predominantly β -sheets (peak $\sim 1631 cm^{-1}$) directly within the Alzheimer's diseased tissue.⁵⁶ Further combined with x-ray imaging, a spatial correlation has been observed between elevated β -sheet and accumulated Cu and Zn ions in A β plaques [Fig. 3(A)].⁵⁷ The protein density of the amyloid plaque was estimated to be approximately 1.6 times greater than that of surrounding brain tissue.⁵⁸ The use of the focal plane array, which is a multi-element detector, largely improved the imaging speed and throughput, enabling more applications. For instance, using a synchrotron IR microscope with the focal plane array, increased lipid contents were

TABLE I Amide I band is sensitive to protein secondary structure in infrared and Raman spectroscopy.

Protein secondary structures and corresponding range for component peaks (cm ⁻¹)				
	α -helix	β -sheet	β -turn	disordered
Infrared ^a	1647–1668	1600–1645, 1682–1699	1668–1682	1645–1647
Raman ^b	1649–1660	1620–1648, 1665–1680	1658–1680, 1680–1699	1660–1665

^aFrom Ref. 12 after reviewing Refs. 28–30 and 38–40.
^bFrom Ref. 41 validated by circular dichroism and x-ray, additional Refs. 31, 34, and 42–44.

detected surrounding—as well as within—the dense core of amyloid plaques [Fig. 3(B)].^{59,60}

Time-lapsed IR imaging in live cells

Further coupled with an IR-compatible environmental chamber, time-lapsed infrared imaging was achieved in a live cell model of amyotrophic lateral sclerosis (ALS). Chinese hamster ovary (CHO-K1) cells were transfected to express a mutant of superoxide dismutase (SOD1) with yellow fluorescent protein (YFP). IR imaging performed 11–16 h post-transfection showed a shift of the amide I band to higher wavenumbers, suggesting an increase in anti-parallel β -sheet structure, consistent with the formation of pore-forming oligomeric structures [Fig. 3(C)].⁵⁵ Another time-resolved FTIR micro-spectroscopy monitored the protein response to severe heat shock (from 37 to 42 °C) in live cells and observed intracellular accumulation of extended β -folded protein aggregates after 40 min using the second derivatives of the spectra and 2D correlation analysis [Fig. 3(D)].⁶¹

BIOMOLECULAR ASSEMBLY BY MID-INFRARED PHOTOTHERMAL MIP)-BASED APPROACHES
Mid-infrared photothermal MIP)/optical photothermal infrared O-PTIR) microscopy

Mid-infrared photothermal (MIP) microscopy, also referred to as optical photothermal infrared (O-PTIR) microscopy, is the latest advancement of vibrational spectroscopic imaging. Instead of directly detecting infrared light and being limited by long wavelengths, MIP introduced a second visible probe to detect the transient changes induced by absorbing pulsed infrared light, including changes in scattering, fluorescence intensity, and refractive index. This approach significantly improved the spatial resolution to the sub-micrometer level and enabled various applications in living biological systems [Fig. 4(A)].^{62,63}

MIP spectroscopy of the amide I region for protein aggregation

The application of MIP to study protein aggregation quickly gained interest due to the potential of explaining structural changes that occur in live cells modeling neurodegenerative diseases. Indeed, the amide I band has strong MIP signals and can report on changes in protein secondary structure. The first demonstration was in primary neurons cultured with synthetic A β _{1–42}, where β -sheet aggregates were identified by calculating the intensity ratio of the 1630 cm⁻¹ peak over the 1650 cm⁻¹ peak at the

subcellular level [Fig. 4(B)]. This work reported structural polymorphisms of amyloid aggregates in AD transgenic neurons, suggesting different mechanisms of AD progression could be triggered by structural polymorphism in the early stage as in neurons.⁶⁴ OPTIR was further combined with synchrotron-based x-ray fluorescence nano-imaging techniques to show that iron clusters co-localize with elevated levels of amyloid β -sheet structures and oxidized lipids in primary AD-like neurons.⁶⁵

MIP coupled with fluorescence imaging

Fluorescence imaging and MIP imaging are highly compatible, and fluorescence can be used as a guide for spectral analysis of specific disease-related proteins. For instance, by incorporating fluorescence and mid-infrared spectroscopy into intensity diffraction tomography, 3D visualization of intracellular tau fibrils by β -sheet structures was demonstrated in human cells seeded with extracted tau fibril fractions [Fig. 4(C)].⁶⁶ O-PTIR was also combined with wide-field epi-fluorescence to perform spectroscopic analysis with the guidance of fluorescent amyloid tracers or amyloid-specific antibodies [Fig. 4(D)].⁶⁷

Label-free identification of protein aggregates by -sheet enrichment

More recently, we demonstrated in live cell modeling Huntington’s disease that β -sheet enrichment could be detected in huntingtin (htt) aggregates.¹² Based on spectral features established with fluorescent guidance, we achieved label-free identification of htt protein aggregates and demonstrated perturbation to the secondary structure caused by the fusion of fluorescent proteins. We also reported the spatial partition of a β -sheet-rich core and an α -helix-rich shell for htt inclusions, a feature that only existed in the [RNQ⁺] prion state but not [rnq⁻] [Fig. 4(E)],¹² confirming a key role of this prion in the aggregation of htt in yeast cells.^{13,68} This demonstrates how MIP can introduce another layer of structural information over morphological imaging data.

BIOMOLECULAR ASSEMBLY BY RAMAN-BASED APPROACHES
Spontaneous Raman spectroscopy and in vitro applications

Chemical bond information is not only encoded by infrared absorption but also by Raman scattering. Raman effect is the

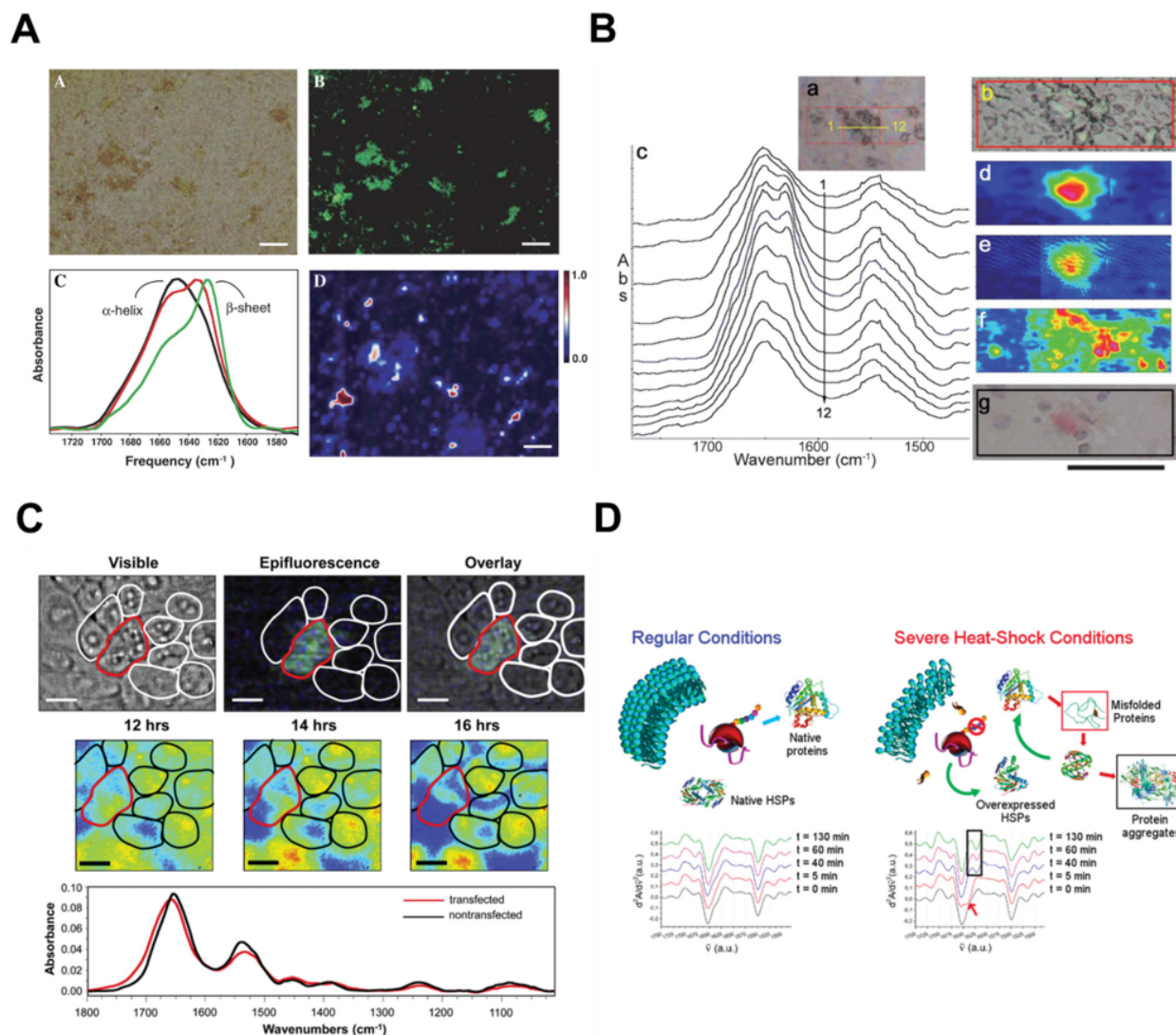


FIG 3 Study of brain tissues and time-lapsed imaging by synchrotron-based IR microscopes. A) Amyloid plaques in human AD tissue show elevated β -sheet and accumulation of Cu and Zn ions. a) bright field; b) Thioflavin S staining of amyloid plaques; c) infrared spectra of Thioflavin-positive (red), Thioflavin-negative (black), and purified A β peptide *in vitro* (green). d) synchrotron x-ray fluorescence spectra of Thioflavin-positive (red) and Thioflavin-negative (black). Scale bar 100 μ m. Reproduced with permission from Miller *et al.*, J. Struct. Biol. **155** (1), 30–37 (2006).⁵⁷ Copyright 2006 Elsevier. B) Increased lipid contents were detected surrounding and within the dense core of amyloid plaques. a and b) bright field; c) IR spectra along the yellow line in a); d) amide I β -sheet 1630 cm^{-1} ; e) plaque density 1390 cm^{-1} ; f) symmetric CH₂ stretching mainly from lipids 2850 cm^{-1} ; g) stained after FTIR imaging with Congo red (plaque core) and Hematoxylin blue (nuclei). Scale bar 50 μ m. Reproduced with permission from Liao *et al.*, Analyst **138** (14), 3991–3997 (2013).⁶⁰ Copyright 2013 The Royal Society of Chemistry. C) An increase in anti-parallel β -sheet structure was detected in live cells expressing mutant SOD1-YFP, suggesting the formation of oligomeric structures. All scale bars are 10 μ m. Reproduced with permission from Miller *et al.*, Biochim. Biophys. Acta **1828** (10), 2339–2346 (2013).⁵⁵ Copyright 2013 Elsevier. D) β -sheet detected in live cell 40 min responding to severe heat shock. Reproduced with permission from Mitri *et al.*, Anal. Chem. **87** (7), 3670–3677 (2015).⁶¹ Copyright 2015 American Chemical Society.

inelastic scattering of photons. Most photons are elastically scattered (Rayleigh scattering), meaning energy and frequency (or wavelength) remain the same. However, an extremely small fraction of photons are scattered inelastically (Raman scattering), where the molecule gains vibrational energy and the scattered photons carry lower energy [Fig. 5(A)]. Raman spectroscopy has been

broadly utilized for the structural characterization of amyloidogenic proteins, prefibrillar oligomers, and mature fibrils *in vitro*.³⁴ One example studied the structural features of α -synuclein fibrils with Parkinson's disease related mutations and showed spectral differences in amide I, amide III, and fingerprint regions [Fig. 5(B)].⁶⁹

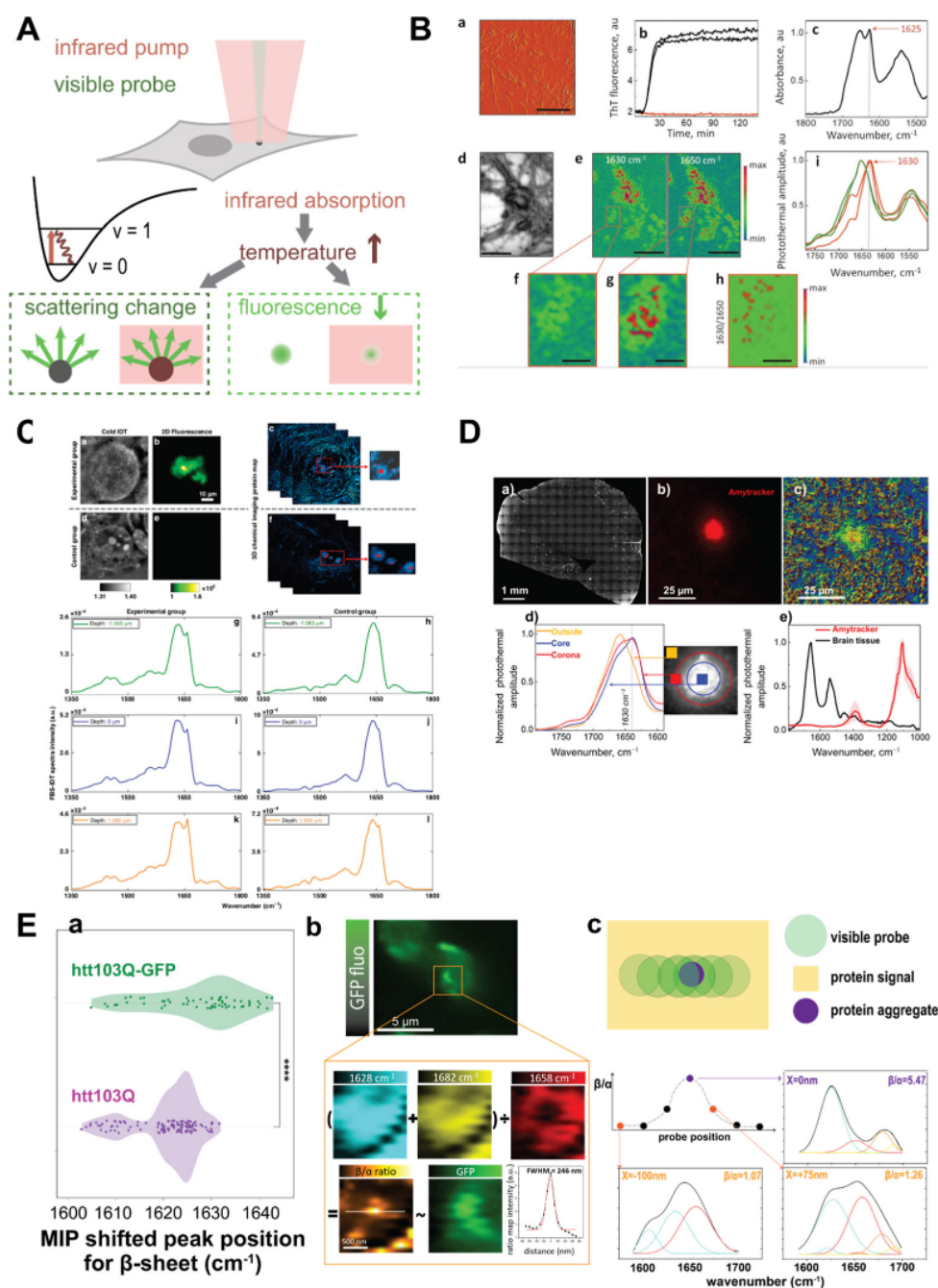


FIG 4 Mid-infrared photothermal spectroscopy and imaging of protein aggregation. A) Principle of mid-infrared photothermal, or optical photothermal infrared microscopy. B) Mapping β -sheet aggregation by intensity ratio of the 1630 cm^{-1} over the 1650 cm^{-1} in primary neurons cultured with synthetic $A\beta_{1-42}$. Scale bars in f) g) and h) is $5\text{ }\mu\text{m}$. Reproduced by permission from Klementieva *et al*, *Adv. Sci.* **7** (6), 1903004 (2020).⁶⁴ Copyright 2020 Wiley. C) 3D visualization of intracellular tau fibrils by β -sheet structures, guided by fluorescence. Reproduced with permission from Zhao *et al*, *Light* **12** (1), 147 (2023).⁶⁵ Copyright 2023 Springer Nature. D) Amyloid plaques in brain tissue labeled with Amytracker 520 with OPTIR imaging and spectra. Reproduced with permission from Prater *et al*, *J. Med. Chem.* **66** (4), 2542–2549 (2023).⁶⁷ Copyright 2023 American Chemical Society. E) Structural mapping of htt aggregates a) compared with htt103Q-GFP aggregates identified by fluorescent guidance, htt103Q aggregates identified label-free by β -sheet enrichment shifted further toward low wavenumbers; b) htt103Q-GFP aggregate partitions into α -helix dominant core and β -sheet shell; c) β -sheet enrichment quantified by deconvolution of amide I band of MIP spectra by small spatial distances within the aggregation complex. Reproduced with permission from Guo *et al*, *Angew. Chem., Int. Ed.* **136**, e202408163 (2024).¹² Copyright 2024 Wiley.

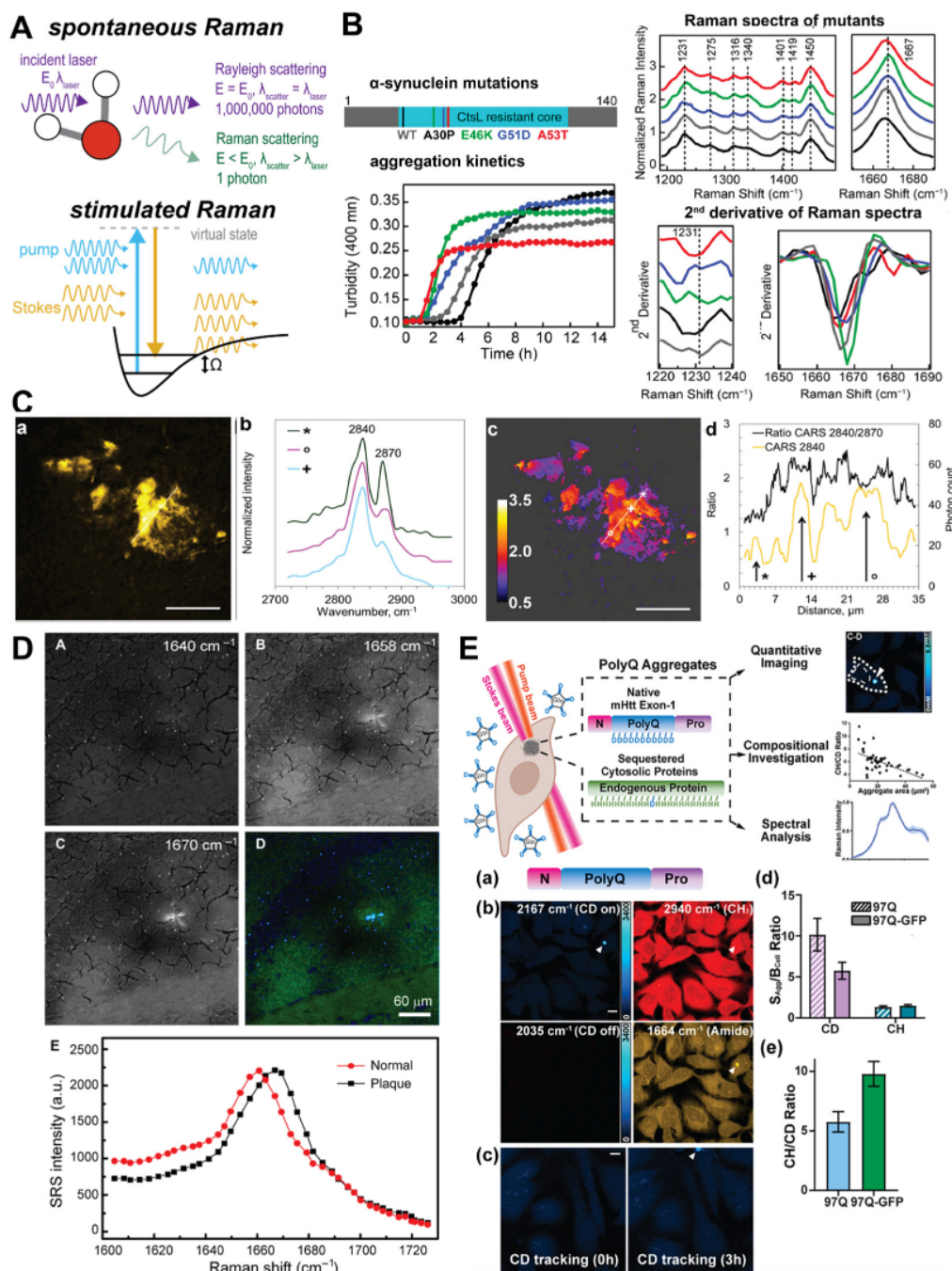


FIG 5 Spontaneous and stimulated Raman spectroscopic studies of protein aggregations. A) principle of spontaneous and stimulated Raman scattering. Spontaneous Raman spectroscopy detects inelastically scattered photons (in green) from the incident photon (in purple). The energy difference between pump (in blue) and Stokes (in orange) matches the energy of the vibrational mode. B) Raman spectroscopy of α -synuclein fibrils with Parkinson's disease-related mutations. Adapted with permission from Flynn *et al.*, J. Biol. Chem. **293** (3), 767–776 (2018).⁶⁹ Copyright 2018 Elsevier. C) CARS and 2P fluorescence imaging of Alzheimer's disease human brain tissue stained with Thioflavin S. Scale bars, 25 μm . Reproduced with permission from Kiskis *et al.*, Sci. Rep. **5** (1), 13489 (2015).⁷⁰ Copyright 2015 Springer Nature. D) Label-free imaging of amyloid plaques in AD by SRS based on the blue shift of the amide I band. Reproduced with permission from Ji *et al.*, Sci. Adv. **4** (11), eaat7715 (2018).⁷¹ Copyright 2018 American Association for the Advancement of Science. E) Selective labeling of htt aggregates by deuterated glutamine with and without tagging GFP. Scale bar: 10 μm . Reproduced with permission from Miao and Wei, ACS Cent. Sci. **6** (4), 478–486 (2020).⁷³ Copyright 2020 American Chemical Society.

Coherent Raman scattering microscopy

Spontaneous Raman scattering is a very rare event (about 1 in 1×10^6 photons), leading to long integration time for the good-quality single-point spectrum. Coherent Raman scattering microscopy was developed to circumvent this challenge. Two powerful lasers are used, called a pump (higher photon energy) and Stokes (lower photon energy). When the energy difference between pump and Stokes matches the energy of the vibrational mode, a pump photon converts to a Stokes photon, leading to a small gain in Stokes intensity and the corresponding loss in pump intensity [Fig. 5(A)]. Coherent Raman scattering microscopy, including coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), significantly improved the sensitivity and imaging speed, enabling diverse biomedical applications.²⁷

Alzheimer's disease brain tissues by CARS and SRS

Alzheimer's disease brain tissue was one of the early applications of coherent Raman scattering microscopy. By coupling CARS and 2-photon fluorescence microscopy for Thioflavin-S staining, lipid deposits were also shown to co-localize with fibrillary β -amyloid plaques, and lipid composition/organization varies throughout the plaques [Fig. 5(C)].⁷⁰ Label-free imaging of amyloid plaques in Alzheimer's disease was achieved with SRS based on the blue shift ($\sim 10 \text{ cm}^{-1}$) of the amide I band originating from the enriched β -sheet content [Fig. 5(D)].⁷¹

Huntington's disease cellular models by SRS

Another set of demonstrations of SRS for studying protein aggregation was based on cellular models of Huntington's disease. SRS was combined with ¹³C-phenylalanine labeling (shift from 1004 to 968 cm^{-1} due to isotopic effect) to visualize protein degradation in live cells and was applied to the htt94Q-mEos2 aggregation process in HEK293T cells.⁷² Selective labeling was achieved by culturing cells with deuterated glutamine given the poly-glutamine nature of mutant htt proteins, and aggregates were identified by imaging carbon-deuterium (C-D) without tagging GFP [Fig. 5(E)].⁷³ More recently, combining SRS and expansion microscopy, the newly synthesized protein was visualized in HeLa cells expressing htt74Q-GFP by deuterated amino acid labeling.⁷⁴

DISCUSSION

Building upon extensive *in vitro* spectroscopic studies, advanced vibrational spectroscopic imaging has been successfully applied to multiple disease-related models of protein aggregation with sub-micrometer spatial resolution, in tissues and cells, especially live cells. In this section, we discuss some of the future directions in terms of biomolecular assemblies, and the technical bottlenecks and ongoing efforts to image these structures with these techniques. We also discuss potential new opportunities ushered in by technical innovations, as well as gaps remaining to be addressed, from a more general perspective [Fig. 6(a)].

From aggregates to condensates. Amyloid aggregates and dynamic condensates are both potentially good targets for vibrational techniques due to local high protein concentrations. They could be distinguished from one another due to differences in their secondary structures, such as the strong β -sheets signature of amyloid aggregates.

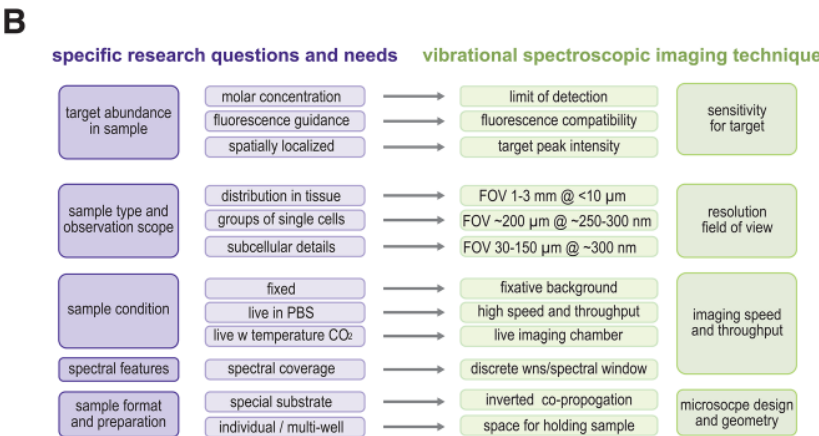
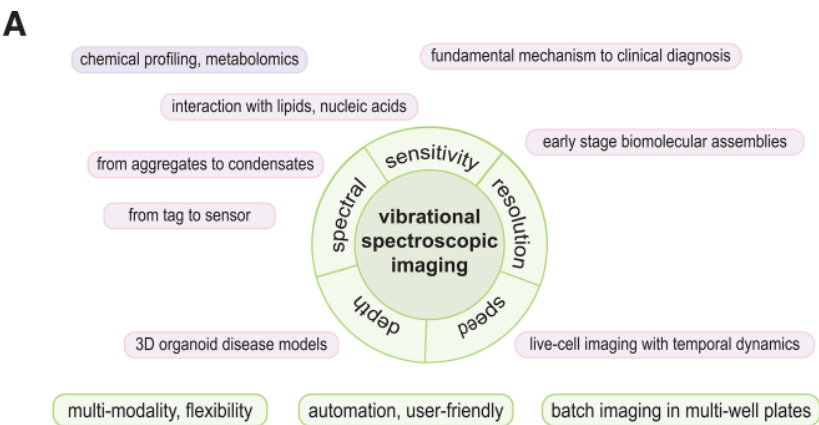
Interaction with lipids or nucleic acids. Direct visual evidence of lipids or nucleic acid involvement in protein aggregates and

condensates could provide new insights. For example, high-content SRS imaging enabled simultaneous mapping of five major biomolecules.⁷⁵ Quantifying different biomolecules in multi-species assemblies could be possible.

Chemical profiling metabolomics. To expand to a broader scope, currently, the major classes of biomolecules were analyzed by their common chemical structures, benefiting from strong signals. However, it would be of interest to deep dive into the specific subtypes, for example, different phospholipids. Differentiating subtypes require scrutiny over the subtle differences in spectrum, possibly by merely one chemical group in structure. For example, the chain length and unsaturation level of fatty acid has been characterized in engineered *E. coli* strains.⁷⁶ On the other hand, live-cell metabolomics has been explored by a library of biorthogonal IR and Raman probes tracking multiple metabolic pathways,⁷⁷ as well as efforts to link with other multi-omics by single-cell Raman spectra,⁷⁸ and with machine learning.⁷⁹

Live-cell imaging of temporal dynamics. Several groups have noted the fluorescent⁸⁰ and spectral differences in biomolecular assemblies between fresh and fixed tissue,^{71,81} or live and fixed cells,^{12,52,73,82} suggesting that live imaging would provide a more faithful representation of structural components. Live-cell imaging would at least have these two advantages: (i) signal would not be confounded by the fixation process; (ii) measurements with temporal resolution would elucidate the dynamics in structural and compositional changes as biomolecular assemblies transition between different states. To achieve this, a suitable environment for cell growth is necessary. Longitudinal single-cell imaging of engineered *E. coli* strains has been achieved to visualize free fatty acid chain length and unsaturation levels over many cycles.⁷⁶ A flexible chamber was designed for time-lapse live-cell SRS imaging, and it demonstrated imaging SKOV3 cells for up to 24 h.⁸³ Integration of an incubation chamber with a MIP microscope also enabled visualization of cell division by cytoplasmic protein.⁸⁴ Finally, a boost in hyperspectral imaging speed is required to capture faster dynamics within minutes. Metabolic dynamics from deuterium oxide (D_2O) have been demonstrated within 1 min in mouse ear skin and *C. elegans* at single color.⁸⁵ Lipid droplet dynamics have been captured within seconds by microsecond fingerprint SRS imaging⁸⁶ and video-rate MIP imaging.⁸⁷ Continued technical advancement is pushing live-cell imaging beyond video rate.⁸⁸

Detect early-stage biomolecular assemblies. Detecting different stages of biomolecular assemblies is vital for understanding their formation process. Amyloid proteins first assemble into oligomers, and oligomers further develop into mature fibrils.¹⁷ Lately, oligomers have attracted attention as potentially the true causes of toxic pathological manifestations.⁸⁹ However, the extremely small size, structural heterogeneity, and metastability of oligomers require higher detection sensitivity and spatial resolution. Instrumental development has been pushing the limit of spatial resolution. For example, near resonance enhanced SRS improved resolution to 130 nm ,⁹⁰ and the recently developed stimulated Raman photothermal microscopy improved sensitivity.⁹¹ Computational approaches can break physical barriers. For instance, a self-supervised deep learning denoiser tailored for vibrational hyperspectral imaging enabled the mapping of low-concentration molecules.⁹² An ongoing pursuit of nanoscopy for vibrational imaging has pushed the resolution below 100 nm ⁹³ and showcased virus replication sites within the nucleus.⁹⁴ These technical



C

	spectroscopy	single-cell imaging	sub-micrometer imaging	cutting-edge imaging
Infrared	FTIR Spectrometer	Infrared Microscope	MIP (O-PTIR)	high speed / ultrasensitive / ...
Raman	Raman Spectrometer	Raman Microscope	CARS / SRS	SRS naoscopy / SRP / ...
Availability	mature commercial products shared facilities	mature commercial products dedicated space	commercial products available and upgrading	academic collaboration technical innovations modifications possible
Sample / Application	in vitro studies standard reference	single-cell analysis tissue	subcellular features spatial distribution	live-cell dynamics, low abundance, subtle differentiation, ...

FIG 6 a) Illustration for future directions in imaging biomolecular assemblies, technical innovations and remaining gaps. b) Schematic illustration of specific research questions and available vibrational spectroscopic imaging techniques that could be used to address them. c) Reference table for implementing vibrational spectroscopic tools. SRP: stimulated Raman photothermal.

advances hold promise for detecting early-stage biomolecular assemblies, thus providing new insights into aggregation mechanisms.

3D organoid disease models: More complicated disease models could better recapitulate pathologies, such as bigenic (amyloid- β , -synuclein and amyloid- β , Tau) cellular models⁹⁵ and human-derived 2D and 3D culture systems.⁹⁶ Very recently, SRS imaging with cell-type specific staining observed *in situ* significant lipid droplet accumulation in microglia of tauopathy mouse brains, while human iPSC-derived neurons with frontotemporal dementia (FTD)-causing V337M Tau mutation accumulate lipid droplets *in vitro* and transfer to glia via neuronal conditioned medium.⁹⁷ Shifting from 2D imaging to image a 3D space poses new challenges to the imaging depth.

Recently, new modality shortwave infrared photothermal microscopy has reached millimeter depth with micrometer resolution, and is particularly suitable for lipids.⁹⁸

From tag to sensor: Tagging with biorthogonal chemical groups could also serve as chemical sensors, as their peak position could shift due to changes in the local environment, such as binding to enzyme⁵⁰ and via the vibrational Stark effect.⁹⁹

Fundamental mechanism to clinical applications. Close collaboration with vibrational spectroscopic imaging tool developers could enable researchers to explore various aspects of biomolecular assemblies, hopefully shedding new light on the fundamental mechanisms of neurodegenerative diseases and identifying novel routes for clinical

applications, such as early detection of amyloid from cerebrospinal fluids, or quantification of highly correlated metabolites from blood.

While we hold positive visions toward the future, it is important to discuss directions requiring more commercialization efforts to facilitate wider application of vibrational spectroscopic imaging.

Multi-modality flexibility. One of the most common concerns for potential users is which modality of the many vibrational spectroscopic imaging tools would best suit their specific research needs. We propose a series of questions bridging the research needs and tool options for reference [Figs. 6(b) and 6(c)]. However, the ideal solution would be to integrate multiple modalities into the same microscope to offer users the flexibility to easily conduct preliminary experiments and guide next steps with data. IR-Raman dual modality could provide complementary information and validate each other, especially in exploratory studies. For example, FTIR and Raman imaging of different types of A β plaque (diffuse, compact, and classical cored) revealed the increase in A β fibrillation alongside the plaque development sequence.¹⁰⁰ Dual modality systems are being developed currently, such as the Raman integrated mid-infrared photothermal microscope¹⁰¹ and the MIP-SRP dual system.¹⁰² On the other hand, high compatibility with fluorescence provides not only better target specificity but also an orthogonal validation. Instrumentation-wise, the same lasers usually double as fluorescence excitation sources and provide naturally co-registered dual-modality images with matching resolution. Alternatively, fluorescence can also be used as a thermal sensor to infrared induced changes.^{12,103–105} Further integration with fluorescence lifetime imaging microscopy (FLIM), fluorescence recovery after photobleaching (FRAP), and fluorescence resonance energy transfer (FRET) could further provide extra information.

Automation user-friendly. A user-friendly system that automates the process pipeline would be appealing to a broad range of researchers. One of the most confusing parts for new users is how to distinguish the target concentration distribution from the raw stack of images. This process has been heavily dependent on expertise, and approaches vary between research groups. A generalizable framework tailored to vibrational spectroscopic imaging takes care of steps like denoising, spectral unmixing, referencing with fluorescence images, and presents directly to users the final color-coded target concentration images.

Batch Imaging in multi-well plates. Comparison between different groups and replicates within groups is the common requirement for researchers. However, the tight space in advanced vibrational spectroscopic imaging tools often restricts to manual switching of individual samples. Recent innovation with long working distance¹⁰⁶ opened opportunities for batch imaging in the form of multi-well plates would be preferred with high consistency between samples.

In conclusion, the recent developments in vibrational techniques have attracted wide interest from the research communities in neurodegenerative diseases due to their recent application in the study of protein aggregates in live cells or tissue samples. However, these advances are only exploring a limited space in the potential application of vibrational techniques in the study of protein condensation and formation of macromolecular assemblies in biology and diseases.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Zhongyue Guo: Conceptualization (equal); Investigation (equal); Supervision (equal); Visualization (lead); Writing – original draft (equal); Writing – review editing (equal). **Giulio Chiesa:** Conceptualization (equal); Investigation (equal); Visualization (supporting); Writing – original draft (equal); Writing – review editing (equal). **Ji-Xin Cheng:** Conceptualization (equal); Investigation (equal); Supervision (equal); Visualization (supporting); Writing – original draft (equal); Writing – review editing (equal).

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed.

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