Present and Future Opportunities in Imaging the Ubiquitin System (Ub-System)

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Present and Future Opportunities in Imaging the Ubiquitin System (Ub-System)

Leonardo Mortati [1,*], Barbara Pergolizzi [2], Cristina Panuzzo [2] and Enrico Bracco [3,*]

[1] INRIM-Istituto Nazionale di Ricerca Metrologica, 10135 Turin, Italy
[2] Department of Clinical and Biological Sciences, University of Turin, 10043 Orbassano, Italy; barbara.pergolizzi@unito.it (B.P.); cristina.panuzzo@unito.it (C.P.)
[3] Department of Oncology, University of Turin, 10043 Orbassano, Italy
* Correspondence: l.mortati@inrim.it (L.M.); enrico.bracco@unito.it (E.B.)

Abstract: From yeast to mammalian cells, ubiquitination is one of the most conserved, and reversible, eukaryotic post-translational modifications (PTMs) responsible for controlling nearly all cellular processes. Potentially, every single eukaryotic cell can accomplish different ubiquitination processes at once, which in turn control the execution of specific cellular events in time and space with different biological significance (e.g., protein degradation or protein–protein interaction). Overall, all these signals are highly dynamic and need to be finely integrated to achieve a proper cellular response. Altogether, ubiquitination appears to be an extremely complex process, likely more than any other PTMs. Until a few years ago, the prevailing experimental approaches to investigate the different aspects of the ubiquitin system entailed genetic and biochemical analysis. However, recently, reagents and technologies have been developed enabling microscopy-based imaging of ubiquitination to enter the scene. In this paper, we discuss the progress made with conventional (confocal fluorescence microscopy) and non-conventional non-linear microscopy (Atomic Force Microscopy—AFM, Coherent Anti-Stokes Raman Scattering—CARS, Stimulated Raman Scattering—SRS) and we speculate on future developments.

Keywords: Ub system; super-resolution microscopy (SRM); atomic force microscopy (AFM); coherent anti-Stokes Raman scattering (CARS); stimulated Raman scattering (SRS)

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1. Introduction

From yeast to mammalian cells, ubiquitination is one of the most conserved, and reversible, eukaryotic post-translational modifications (PTMs) responsible for controlling nearly all cellular processes [1–3], including transcription by controlling DNA-RNA hybrid (R-loops) levels through a macromolecular complex, the degradosome [4]. Ubiquitination entails the synthesis of a covalent isopeptide bond between the C-terminal Gly residue of the ubiquitin (Ub) and the protein substrates [5]. Ubiquitin is a highly conserved eukaryotic protein composed of 76 amino acids with a molecular weight of 8.5 kDa and dimensions of 5.1 × 4.3 × 2.9 nm (https://www.rcsb.org/structure/1UBQ (accessed on 21 June 2022)). Ubiquitination controls the protein fate and thus significantly contributes, alongside protein synthesis, to cellular protein homeostasis (proteostasis) [6]. The control can occur by targeting a protein for proteolysis through the ubiquitin–proteasome system (UPS), hence shortening its half-life. Alternatively, a Ub-tagged protein might display an altered subcellular localization and/or activity. The required instructions for determining the destiny of the Ub conjugates are represented by a very complex, though finely tuned, Ub code whose biological meaning has started to be deciphered in recent years. Indeed, Ub can be conjugated to substrates as a monomer on one or more sites, giving rise to monoubiquitylation or multi-monoubiquitylation, respectively. Additionally, through one of the seven lysine residues or via the first methionine, the Ub monomers can be

polymerized to form distinct kinds of chains, which are classified into two main categories according to the different types of linkages linking the nearby Ub monomers within the chain. Homotypic chains are those in which the Ub monomers are linked uniformly through the same acceptor site. Conversely, heterotypic chains are linked through multiple sites and can be further sub-categorized into mixed or branched. Mixed chains consist of more than one type of linkage, but each ubiquitin monomer within the chain is modified on only a single acceptor site. By contrast, branched chains comprise one or more ubiquitin subunits that are concurrently modified on at least two different acceptor sites [7,8]. For the correct interpretation of the Ub code, the cells require "decoders/readers" represented by an array of ubiquitin binding domain (UBD)-containing proteins [9]. In addition to the role played by the Ub conjugates, the maintenance of a free Ub pool and cytosolic unanchored poly-Ub chains is equally important for cellular functions and survival [10]. The latter is emerging as a key factor in multiple cellular responses, including innate antiviral pathways and kinase activity, among others [11,12]. Free poly Ub chains can also activate the aggresome pathway, another mechanism that degrades unwanted proteins to overcome proteasome overwhelming or inhibition [13].

Potentially, every single eukaryotic cell can accomplish different ubiquitination processes at once, which in turn control the execution of specific cellular events in time and space with different biological significance (e.g., protein degradation or protein–protein interaction). Overall, all these signals are highly dynamic and need to be finely integrated to achieve a proper cellular response. Altogether, ubiquitination appears to be an extremely complex process, likely more than any other PTMs.

Hence, aberrations in the ubiquitin system result in a wide range of disorders, including developmental diseases, cancer, and neurodegeneration [2]. Abnormal misfolded protein disposal, due to impairment in the ubiquitin system activity, has detrimental effects on intracellular processes and eventually cell function and has been associated with several neurodegenerative diseases and aging. Indeed, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and the polyglutamine diseases, including Huntington's disease, typically involve deposits of inclusion bodies, composed of misfolded proteins, in the brain's cells [14]. Amazingly, intracellular protein aggregates impair proteasome activity, thus leading to a degenerative loop [15].

Up until a few years ago, the prevailing experimental approaches to investigate the different aspects of the ubiquitin system entailed genetic and biochemical analysis. Even though these approaches have progressed tremendously, including the computational methods aiming to understand the dynamics of proteins and bio-macromolecular complexes in solution [16], they display a common limitation. Indeed, they do not allow us to visualize the dynamics of protein complexes on a whole cell or tissue. However, in recent times, we have witnessed the development of reagents and technologies enabling microscopy-based imaging of ubiquitination. In this paper, we discuss the progress made with conventional (confocal fluorescence microscopy) and non-conventional non-linear microscopy (Atomic Force Microscopy—AFM, Coherent Anti-Stokes Raman Scattering—CARS, Stimulated Raman Scattering—SRS) and we speculate on future developments.

2. Approaches to Study Protein Ubiquitination

2.1. Biochemical and Genetic Approaches

Previously, the different aspects and questions regarding ubiquitination were addressed almost exclusively by using biochemical and genetic methods. Nowadays, biochemical experiments are still the unsurpassed method to identify potential ubiquitinated substrate(s), assess the Ub chain specificity, and discriminate between mono- and polyubiquitination. The biochemical approaches can be applied to a range of biological materials, including in vitro ubiquitination reactions, cellular lysates, whole tissues, and eventually also to organisms. However, biochemistry-based methods suffer from several limitations because measurements occur on cell lysates, thus potentially increasing the incidence of artifacts. More importantly, protein interactions may often be too weak to be detected by

pull-down assay and Western blotting. The restriction of Ub reactions to specific cellular compartments, or subsets of targets, often requires cell fractionation to enrich specific substrate or chain types. Scaling up to high-throughput or high-content settings is also quite challenging, providing a rather scant spatio-temporal resolution. Genetics, especially in simple model systems, significantly contributed to the identification and functional characterization of many components (E1, E2, E3, DUBs, and UIMs and substrates) of the ubiquitin system [17–19]. Eventually, semi-high-throughput yeast two-hybrid analysis enabled the unraveling of the complex network underlying the E2–E3 interaction and determining the E3 substrate(s) [20,21].

2.2. Microscopy Approach

In recent years, biochemical methods have been remarkably implemented with the help of imaging-based approaches. The reason is largely due to the development, and applications, of novel reagents enabling the visualization of the different ubiquitin chains down to a subcellular level. The development of microscopy techniques achieving a high spatial resolution, alongside non-conventional and non-linear microscopy, which combine microscopy and optical spectroscopy, thus enabling a direct, non-invasive, and label-free imaging of biological macromolecules, would enormously facilitate the depiction of a sharper picture of the ubiquitin system dynamics. While conventional optical microscopy would help to examine the dynamics of the ubiquitin system, non-conventional (e.g., AFM) and non-linear microspectroscopy (e.g., CARS and SRS) would facilitate the assessment of the changes in physicochemical, including mechanical, properties occurring when the ubiquitin system is malfunctioning and leading to aberrant protein accumulation and aggregates, either at the tissue, cellular, or subcellular level.

2.2.1. Indirect Imaging of Ubiquitination in Protein Degradation

In the past, fluorescently labeled, degradation-sensitive reporters have been developed and applied either to microscopy imaging and/or to flow cytofluorimetry [22]. They are based on green fluorescent protein (GFP) and suitable to be utilized when expressed in isolated cells or intact organisms [23–25]. Although these techniques allow the quantification of the reporter stability, they do not allow the direct imaging of the degradative Ub signals. For this reason, this approach is only suitable for imaging degradative functions of Ub in well-characterized biological scenarios that rely on known and well-studied substrates, E3 ligases, and molecular mechanisms. However, the approach centered on reporter-based read-outs presents major flaws when its usage is intended to monitor proteasome-independent functions of ubiquitin (e.g., protein–protein interaction, protein subcellular relocalization, regulation of protein activity, DNA repair, autophagy etc.) [3,26,27] and does not answer questions related to the specific type of ubiquitin modification (e.g., kinds of chains, distinguish between mono and poly-Ub, etc.) [7,28]. Interestingly, non-conventional microscopic approaches were recently set up to assess the physicomechanical changes occurring at molecular and cellular levels in response to the accumulation of misfolded protein aggregates. Additionally, non-linear microscopy techniques enabling high-speed and in vivo label-free imaging of protein aggregates have been successfully applied.

Atomic Force Microscopy reconstructs the morphology of a sample on an atomically flat surface by monitoring the distance-dependent interaction forces between a sharp probe and the external sample surface area. Recently, AFM imaging successfully revealed different dimer morphology associations of thioredoxin enzymes by accurate volumetric studies [29], which could be implemented to analyze ubiquitin polymerization processes. In this respect, AFM has made great strides in determining the morphological features of the quaternary assemblies of human mitochondrial apoptosis-inducing factor (hAIF) protein [30]. The advent of AFM has significantly impacted the study of misfolded proteins because they display a tendency to easily form intracellular insoluble aggregates. Initially, AFM was employed at a single-molecule level, where it proved to be remarkably powerful to unravel the molecular and hierarchical assembly of the misfolded species present transiently

during protein aggregation, visualizing their dynamics at the nanoscale and deciphering the mechanistic details of the aggregation of amyloid-forming proteins [31]. Lately, the application of AFM in combination with infrared nanospectroscopy (AFM-nanoIR) has been a breakthrough in the analysis of the chemical properties of heterogeneous protein aggregation at the nanoscale level. AFM-nanoIR is based on a pulsed, tunable IR laser light aligned to the same scanned area by the conventional AFM tip. The photothermal expansion induced by sample absorption causes the vibration of the chemical bonds involved in the scanned sample surface area and their characterization [32]. The AFM-nanoIR technique has been successfully devoted to discern the chemical changes observed by the interaction of amyloidogenic proteins involved in neurodegenerative disorders with peptide inhibitors [33]. The use of this tool can be fully extendable to carry out measurements related to Ub assembly processes.

The loss of proteostasis is considered a hallmark of aging. Indeed, the gradual accumulation of misfolded and aggregated proteins impairs intracellular processes and cell function, thus contributing to multiple age-related diseases [34]. By taking advantage of conventional and non-conventional microscopy, including AFM, it has been lately determined that aging cells display the formation of protein aggregates which are associated with altered physicomechanical features, including cellular stiffness [35]. More recently, the group led by Govindaraju [36] gave a special emphasis to understanding the neuronal cells under amyloid-induced stress conditions and their rescue by de novo designed peptidomimetics inhibitors employing confocal AFM. Amyloid β undergoes aggregation through conformational changes from random coil to α-helix to β-sheet. Neuronal cells are softer when compared to other cells, more than 10 times as compared with keratinocytes, but their physicomechanical features become altered when they are stressed, and this change has also been associated with neurodegeneration [37]. Interestingly, upon Aβ-42 aggregates-induced stress, the neuronal cells become flattened—up to one-third when compared to the control—rougher, and display extraordinarily augmented cellular stiffness [36].

Raman scattering, either as SRS (Stimulated Raman Scattering) or as CARS (Coherent Anti-Stokes Raman Scattering), has been widely used for the spectroscopic study of biomolecules, especially lipids. The Raman scattering signal can be improved by some orders of magnitude with a non-linear optical method called CARS microscopy [38]. Indeed, due to the coherent addition of the CARS radiations, the CARS intensity is quadratically dependent on the number of vibrational oscillators in the focal volume. In the samples with a high concentration of vibrational oscillators, the CARS signal is many folds stronger than the spontaneous Raman scattering signal, which is linearly dependent on the number of vibrational oscillators. The large signal level in CARS microscopy enables high-speed imaging, which is important for live cell and tissue studies. The high imaging speed allows real-time inspection of biological processes. Moreover, the non-linear optical excitation ensures that the CARS signal is only generated at the center of the focus, thus offering an inherent 3D spatial resolution. With the developments of CARS imaging platforms, high-speed three-dimensional chemical imaging of living biological systems has been widely demonstrated [39–41], also in time-lapse experiments [42].

Consistently, Perney and co-workers [43] made strides in translating protein structure data into microscopy techniques. By using CARS coupled with multi-photon fluorescence microscopy, the authors managed to image poly-Q protein aggregates in a whole animal organism, such as the worm *C. elegans*. Their findings were consistent with a previous inquiry [44] revealing that the diffuse and early "foci" aggregates differ quite significantly from the mature ones in terms of biochemical structural features. Unfortunately, our current knowledge in terms of Raman and or infrared spectra of purified proteins is not as rich and deep as it is for data collected from NMR and X-ray crystallography. The generation of libraries of Raman spectra of characterized proteins have the potential to enable CARS microscopy to become an invaluable tool for the identification of protein structure in vivo, particularly for misfolded proteins in protein aggregates that are a hallmark of several

age-associated diseases. More recently, a novel platform for live-cell imaging, quantification, and spectral analysis of polyQ aggregates by SRS microscopy of deuterium-labeled glutamine has been established [45]. Upon the achievement of precise and sensitive SRS imaging of native polyQ aggregates without the need for large, tagged fusion proteins, the authors developed a remarkable ratiometric strategy to quantitatively analyze the protein content in aggregates of varying sizes. Compared to the currently existing methods, such an approach allows the determination in vivo of the absolute concentrations of both mutant and wild-type Huntingtin (Htt) proteins within the same aggregate in cells. Furthermore, by applying the hyperspectral SRS, the authors probed the intracellular aggregate structures and aggregate–environment interactions (heat shock proteins) upon induced heat shock. Remarkably, for the first time, they succeeded in imaging native polyQ (GFP untagged) protein aggregates, revealing that they are denser, about twice as dense as those in which the polyQ has been tagged with GFP. Overall, the finding indicates that GFP is excellent for in vivo imaging, but in this specific case, because it is quite bulky, it might perturb the polyQ aggregation formation. Notably, using cryo-electron tomography, it has been possible to uncover that mutant Htt (mHtt) aggregates display a fibrillary structure and that mHtt aggregates interact with intracellular membranes, including the endoplasmic reticulum, thus distorting its morphology and dynamics [46]. Besides polyQ aggregates, Raman spectroscopy is a powerful technique to visualize and characterize amyloid peptides. In this respect, multiwavelength Raman spectroscopy has been used to probe the amide bands of the amyloid-β peptide [47], and by taking advantage of an optical spectroscopy and imaging approach based on CARS, the normal amyloid-β regions were distinguished from the abnormal amyloid-β accumulation in murine brain samples [48].

2.2.2. Direct Imaging of Ubiquitination

The imaging, either on fixed cells or in vivo, of ubiquitination by microscopy aims to visualize the local accumulation of different Ub signals. Intriguingly, accumulated Ub-rich structures, such as aggregates, foci, or puncta, are quite easily imaged. Currently, to visualize Ub and Ub structures, there are two different approaches in use: (a) direct, which forecasts the use of Ub point mutants, allowing us to image and characterize the different Ub chains, and (b) indirect, which makes use of reagents recognizing certain types of Ub signals (e.g., UIMs). In this respect, the most popular way to visualize Ub includes the transient or stable (over)expression of tagged or fluorescently labeled Ub. Though such an approach appears straightforward, it presents some serious limitations that must be carefully considered. Indeed, the ectopically expressed labeled Ub must compete with the endogenous one that is robustly expressed. Therefore, sufficient levels of ectopic labeled Ub are required. Conversely, the ectopic expression of Ub on itself might potentially affect cellular processes and the deubiquitination balance. Remarkably, tags and especially the bulky ones (i.e., GFP) might interfere either with the chain growth and/or with the accessibility by different UIMs. Eventually, the expression of labeled w.t. Ub does not give any hints about the type of Ub chain. However, to overcome this hindrance, Ub mutants, in the amino acids (e.g., one of the seven lysine residues) responsible for the Ub monomer polymerization, are helpful [28]. Nonetheless, to some extent, caution should be exercised when interpreting the results because the mutations might force the generation of specific chains. Furthermore, it must also be considered that fusing Ub at its N-terminus with GFP interferes with the ability to form linear chains. However, tracking of specific Ub chains within chains can be overcome by using chain-specific GFP-UBD (green fluorescent protein–ubiquitin binding domain) chimeras [49–51]. Lately, specific probes such as UbDHa (Ub-dehydroalanine) have shown to be suitable and promising tools, coupled to the FRAP and FRET technologies, to quantitatively investigate the cellular and intracellular Ub flux over the E1, E2, and HECT-type E3 ligases [52]. Additionally, a novel developed FRET-based platform has been proven to be suitable to track the substrate ubiquitination by fluorescence, thus providing potential opportunities for advanced reaction dynamic studies [53]. Though this platform allows us to measure ubiquitination in real time, currently, it applies only

to short degron sequences (approximately 20 amino acids) and to a kind of Ub chain that involves the lysine 48 of the Ub moiety (K-48). However, modifications can be made, and the platform has powerful features envisaging its use in high-throughput screening (HTS) campaigns to identify potential small molecule inhibitors and/or activators that target substrate–E3 interaction. The single-molecule Foster Resonance Energy Transfer (smFRET) is one of the most powerful approaches to investigate biomolecule conformations, dynamics, or molecular interaction at a single molecule level. Consistently, the prerequisite to achieve single-molecule sensitivity is that very small concentrations of dye-labeled molecules are required. Recently, approaches aiming to control and measure absolute molecule concentrations have been described [54]. The provided evidence prompt speculation that such an approach might represent a potential and fruitful tool to assess Ub flux. Exceptional levels of spatial resolution of biological molecules and processes can be provided by super-resolution microscopy (SRM) approaches [55,56], whose most common features are shortly summarized in Table 1.

Table 1. Summary of the main features characterizing the different SRM approaches. STED: Stimulated Emission Depletion; RESOLFT: Reversible Saturable Optical Fluorescence Transition; SIM: Structured Illuminated Microscopy; PALM: Photoactivation Localization Microscopy; STORM: Stochastic Optical Reconstruction Microscopy; uPAINT: Universal Point Accumulation Imaging in the Nanoscale Topography. Y: yes; N: no; Y/N: not recommended but possible usage.

	Different Kind of SRM Approaches	Approximate Spatial Resolution	Multicolor Imaging	Intracellular Labeling	Post-Acquisition Images Processing and Analysis	Acquisition Time for Single Image	Dynamics of Large Molecular Structures	Dynamics of Single Molecules
	STED	50–70 nm	Y	Y	N	Medium	Y	Y/N
	RESOLFT	80–100 nm	Y	Y	N	Low	Y	Y/N
	SIM	50–100 nm	Y	Y	Y	Fast	Y	Y/N
Single-molecule approaches	PALM	50 nm	Y	Y	Y	Fast	Y	Y
	STORM	50 nm	Y	Y	Y	Fast	Y	Y
	uPAINT	50 nm	Y	N	Y	Fast	Y	Y

In principle, though to date, most Ub imaging entailed the use of conventional fluorescence microscopy, SRM might represent a privileged tool to image the Ub system at the subcellular level. Indeed, at present, few SRM methods have been successfully employed for imaging ubiquitination, including Structured Illumination Microscopy (SIM), Direct Stochastic Optical Reconstruction Microscopy (dSTORM), and Photoactivated Localization Microscopy (PALM) [57,58]. However, potentially other SRM approaches (e.g., Stimulated Emission Depletion, STED) [59] can also be used for Ub imaging. In this respect, by using the SIM approach in living cells, it has been recently detailed at the molecular level that the amyloidogenic polyglutamine-containing protein first forms small, amorphous aggregate clusters in the cytosol, chiefly by diffusion. Afterwards, the dynamic interactions among these clusters limit their elongation and lead to structures with a branched morphology. Some of these clusters then assemble via active transport at the microtubule-organizing center and thereby initiate the formation of perinuclear protein aggregates in a way that is only partially governed by active transport [60].

3. Conclusions and Perspectives

While progress in the Ub system field has progressed tremendously over the past decade, mostly because of the identification and biochemical characterization of the molecular players, analogous progress in the field of Ub system visualization has only recently started to be appreciated, as summarized in Figure 1.

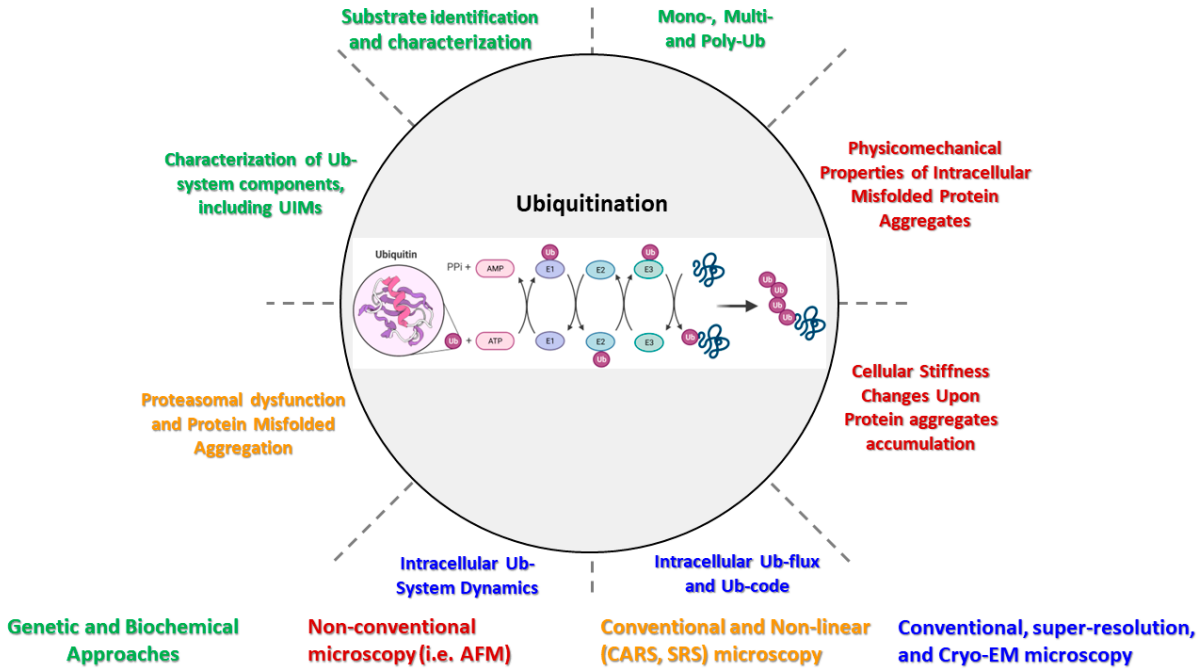

Figure 1. Most common and useful approaches to study the Ub system. Ubiquitination is a highly orchestrated process in which three classes of enzymes, E1, E2, and E3, depicted in the inner core of the figure, are sequentially operating. Since the process is reversible, the ubiquitinated substrate(s) can be either freed from Ub moieties, or the latter can be further modified (e.g., trimmed) by a class of enzymes called deubiquitinating enzymes. Eventually, Ub moieties are recognized, and their meaning decoded by "readers". The process is characterized by being highly dynamic. To date, to address questions regarding the different types of ubiquitination (e.g., mono-Ub vs poly-Ub, linear vs branched Ub chains, etc.) or to characterize the meaning of a particular Ub chain, the biochemical and genetic approaches are preferred (green font color). To assess the extent of Ub system dynamics in cells, the kinetics of misfolded protein aggregates formation, or eventually to probe the viscoelastic changes occurring after intracellular protein aggregates accumulation, different kinds of microscopic approaches have started to be undertaken (dark red, orange, and blue color font). Image created with the help of BIORENDER (https://biorender.com/ (accessed on 20 May 2022)).

Ubiquitination is an extraordinarily and amazingly dynamic process occurring at once in different subcellular compartments; therefore, its imaging represents an undeniable and helpful resource to address some of the open issues in the field of the Ub system. Employing different approaches would enable imaging the complete Ub system with a molecular resolution at a spatial subcellular scale, and hopefully in the near future, this would assist in drawing a subcellular map of the Ub system. Furthermore, the emergence of non-conventional microscopy (i.e., AFM) would be of great benefit for assessing to what degree the malfunctioning of the Ub system, which in turn contributes to the accumulation of intracellular misfolded protein aggregates, might impinge the intracellular architecture, as well as the physicomechanical and viscoelastic properties of the cell, including stiffness anisotropy and heterogeneity. Eventually, this technique would allow us to explore some functional aspects related to the relation with protein aggregates and cytoskeleton as well as various subcellular compartments (i.e., endoplasmic reticulum, mitochondria, lysosomes, etc.). In terms of non-linear optic approaches (i.e., CARS), it is reasonable to assume that a massive boost in the usage of this method is expected, to the extent that Raman libraries of characterized proteins associated with the Ub system, including Ub itself, would be developed and implemented. Moreover, it is plausible to speculate that CARS imaging might represent a profitable technique to examine in vivo the dynamics of lipid droplets autophagy. However, we must be cautious—biochemistry and genetics should not be discarded, as they are fundamental for the development of fine microscopic tools,

especially for SRM. Overall, in the recent past, we have witnessed tremendous efforts aiming to visualize the Ub system at tissue, cellular, and subcellular levels. The integration of the different approaches would enable us to determine the kinetics of the different ubiquitination processes and how they are orchestrated at the cellular level, alongside the accumulation of aggregated misfolded proteins.

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