Visualizing ubiquitination in mammalian cells

Sjoerd JL van Wijk[1],[*] (ID), Simone Fulda[1],[2],[3], Ivan Dikic[4],[5] (ID) & Mike Heilemann[6],[**] (ID)

Abstract

Covalent modification of proteins with ubiquitin is essential for the majority of biological processes in mammalian cells. Numerous proteins are conjugated with single or multiple ubiquitin molecules or chains in a dynamic fashion, often determining protein half-lives, localization or function. Experimental approaches to study ubiquitination have been dominated by genetic and biochemical analysis of enzyme structure–function relationships, reaction mechanisms and physiological relevance. Here, we provide an overview of recent developments in microscopy-based imaging of ubiquitination, available reagents and technologies. We discuss the progress in direct and indirect imaging of differentially linked ubiquitin chains in fixed and living cells using confocal fluorescence microscopy and super-resolution microscopy, illustrated by the role of ubiquitin in antibacterial autophagy and pro-inflammatory signalling. Finally, we speculate on future developments and forecast a transition from qualitative to quantitative super-resolution approaches to understand fundamental aspects of ubiquitination and the formation and distribution of functional E3 ligase protein complexes in their native environment.

See the Glossary for abbreviations used in this article.

Ubiquitination

Major parts of eukaryotic proteomes are controlled and regulated by post-translational modifications and among the most prominent is the covalent modification with the strictly conserved protein ubiquitin (Ub) (ubiquitination or ubiquitylation). Originally identified as a trigger for protein degradation by the 26S proteasome, ubiquitination serves many more proteasome-independent functions, including signal transduction and selective autophagy [1–3].

Ubiquitination thus influences and controls the majority of cellular processes and is implicated in a wide variety of pathophysiological states and diseases, ranging from cancer to infections and hereditary disorders [4].

Ubiquitin is a small, 76-residue regulatory protein that is universally expressed in eukaryotic organisms [2,3]. Ubiquitin is a member of the ubiquitin-like (UBL) protein family and shares sequence and structural homology with proteins like Small Ubiquitin-like Modifier (SUMO) [5,6], Interferon Stimulated Gene 15 (ISG15) [7] and Neural precursor cell Expressed, Developmentally Down-regulated 8 (NEDD8) [8]. Although for many years believed to be strictly expressed in eukaryotes, ubiquitin-like proteins with some similarities in structure and conjugation systems have now been identified in *Mycobacterium tuberculosis* (Pup: prokaryotic ubiquitin-like protein) [9] and in some Gram-negative bacteria (UBact: Ubiquitin Bacterial) [10].

Ubiquitination is mediated by the sequential action of an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin protein ligase (E3) (Fig 1A and B) [3,11–14]. The substrate can be modified with a single ubiquitin (mono-ubiquitination) or with polymeric Ub chains. Depending on which internal lysine (K6, K11, K27, K29, K33, K48, K63) or whether the N-terminal methionine residue (M1, linear or head-to-tail chains) of Ub is used for linkage to the distal Ub different chain types can be generated (Fig 1C and D; Box 1) [3,15,16]. To add complexity, the differential use of Ub lysine residues can generate homotypic chains (linked through one type of residues) or heterotypic or branched chains, such as K63-linear and K48-K11 hybrid polymers, respectively [17,18]. Importantly, the type of ubiquitin signal determines the biological effects of these modifications; for example, K48 and heterotypic K11/K48 chains generally target substrates for degradation by the 26S proteasome. In contrast, chains linked through other residues, like K6, K27, K33, K63 and linear ubiquitin chains, are often involved in non-degradative purposes, like selective autophagy, DNA damage repair and innate immunity [3]. This information is decoded by proteins containing ubiquitin-binding domains (UBDs) that recognize chain-specific residues exposed on proximal and distal ubiquitin molecules and within the linker regions connecting two ubiquitin molecules (Fig 1B) [19–22]. Deubiquitinating enzymes

1 Institute for Experimental Cancer Research in Paediatrics, Goethe University, Frankfurt am Main, Germany
2 German Cancer Consortium (DKTK), Heidelberg, Germany
3 German Cancer Research Centre (DKFZ), Heidelberg, Germany
4 Institute of Biochemistry II, Goethe University – Medical Faculty, University Hospital Frankfurt, Frankfurt am Main, Germany
5 Buchmann Institute for Molecular Life Sciences (BMLS), Goethe University, Frankfurt am Main, Germany
6 Institute of Physical and Theoretical Chemistry, Goethe University, Frankfurt am Main, Germany
  *Corresponding author. Tel: +49 69 6786 6574; E-mail: s.wijk@kinderkrebsstiftung-frankfurt.de
  **Corresponding author. Tel: +49 69 7982 9736; E-mail: heilemann@chemie.uni-frankfurt.de

Glossary

ACTL8	actin-like protein 8
ARIH1	E3 ubiquitin-protein ligase protein ariadne-1 homolog
BiFC	bimolecular fluorescence complementation
CALCOCO2/NDP52	calcium-binding and coiled-coil domain-containing protein 2/Nuclear domain 10 protein NDP52
CCCP	carbonylcyanid-*m*-chlorphenylhydrazon
Cdt1	DNA replication factor Cdt1
CHIP	carboxyl terminus of Hsp70-interacting protein
CLSM	confocal laser scanning microscopy
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats
CRL3^{KLHL21}	cullin RING E3 ligase with the KLHL21 adaptor protein
Cy5	cyanine dye 5
deGradFP	proteasomal degradation of GFP fusions
Dha	dehydroalanine
diGly	lys-ϵ-Gly-Gly
dSTORM	direct stochastic optical reconstruction microscopy
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin protein ligase
E6AP	human papillomavirus E6-associated protein
FC(C)S	fluorescence (cross) correlation spectroscopy
FCCP	carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FK1	anti-ubiquitin antibody FK1
FK2	anti-ubiquitin antibody FK2
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FUCCI	fluorescence ubiquitination cell cycle indicator
GFP	green fluorescent protein
GFPu	16-residue CL1 degron fused to GFP
HECT	homologous to the E6-AP carboxyl terminus
HOIL1	RanBP-type and C3HC4-type zinc finger-containing protein 1
HUWE1	HECT, UBA and WWE domain-containing protein 1
IκBα	NF-kappa-B inhibitor alpha
IKK	inhibitor of nuclear factor kappa-B kinase
INT-Ub.7KR	lysine-less, internally tagged ubiquitin
ISG15	interferon stimulated gene 16
KG	Kusabira-Green
K	lysine
LRSAM1	E3 ubiquitin-protein ligase leucine-rich repeat and sterile alpha motif-containing protein 1
LUBAC	linear ubiquitin chain assembly complex
M(et)	methionine
M1-SUB	Met1-linkage-specific Ub-binder
mKO2	monomeric Kusabira-Orange 2
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NEMO	NF-kappa-B essential modulator
NF-κB	nuclear factor NF-kappa-B
nm	nanometre
NSlmb-vhhGFP4	F-box-anti-GFP nanobody fusion protein
N-terminal	amino-terminal
NZF	Npl4 zinc finger
OPTN	optineurin
OTULIN	OTU domain-containing deubiquitinase with linear linkage specificity
OUT	orthogonal Ub transfer (OUT) method
p65/RelA	nuclear factor NF-kappa-B p65 subunit
PAGFP	photoactivatable GFP
PAINT	point accumulation for imaging in nanoscale topography
PALM	photoactivation localization microscopy
PARKIN	E3 ubiquitin-protein ligase parkin
PINK1	serine/threonine-protein kinase PINK1, mitochondrial
PML	promyelocytic leukaemia protein
PolyUb-FC	polyubiquitin-mediated fluorescence complementation
PROTACs	proteolysis-targeting chimeric molecules
PSF	point-spread function
Pup	prokaryotic ubiquitin-like protein
RBR	RING-in-between-RING
RFP	red fluorescent protein
RING	really interesting new gene
SCF	SKP1-CUL1-F-Box
SCV	salmonella-containing vacuole
SIM	structured illumination microscopy
SLBP	histone RNA hairpin-binding protein
SMLM	single-molecule localization microscopy
Smurf2	E3 ubiquitin-protein ligase SMAD ubiquitination regulatory factor 2
SQSTM1/p62	sequestosome-1
SRM	super-resolution microscopy
STED	stimulated emission depletion
SUMO	small ubiquitin-like modifier
TAB 2	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2
TNFR1	tumour necrosis factor receptor superfamily member 1A
TOM20	mitochondrial import receptor subunit TOM20 homolog
TUBE	tandem ubiquitin-binding entity
UBact	ubiquitin bacterial
UBAIT	ubiquitin-activated interaction trap
UBAN	ubiquitin-binding in ABIN and NEMO
UbDha	Ub-dehydroalanine
UBD	ubiquitin-binding domain
UBE1	ubiquitin-activating enzyme 1
UBE2J2	ubiquitin-conjugating enzyme J2
UbIA-MS	ubiquitin interactor affinity enrichment-mass spectrometry
UBL	ubiquitin-like
Ub-ProT	ubiquitin chain protection from trypsinization
Ub	ubiquitin
UCHL3	ubiquitin carboxyl-terminal hydrolase isozyme L3
UiFC	ubiquitination-induced fluorescence complementation
UIM	ubiquitin-interacting motif
UPS	ubiquitin proteasome system
USP30	ubiquitin carboxyl-terminal hydrolase 30
V$_H$H	single-domain antibody fragments
VPS27	vacuolar protein sorting-associated protein 27
xE1, xE2 and xE3	engineered E1, E2 and E3 OUT enzymes
YFP	yellow fluorescent protein

(DUBs) counterbalance chain-growing capacities by removing ubiquitin modifications (Fig 1B) [23,24]. The concerted interplay of chain/linkage formation, recognition by UBDs and Ub hydrolysis creates dynamic networks that control the distribution of different ubiquitin signals, which in turn regulate a plethora of biological processes within the cell.
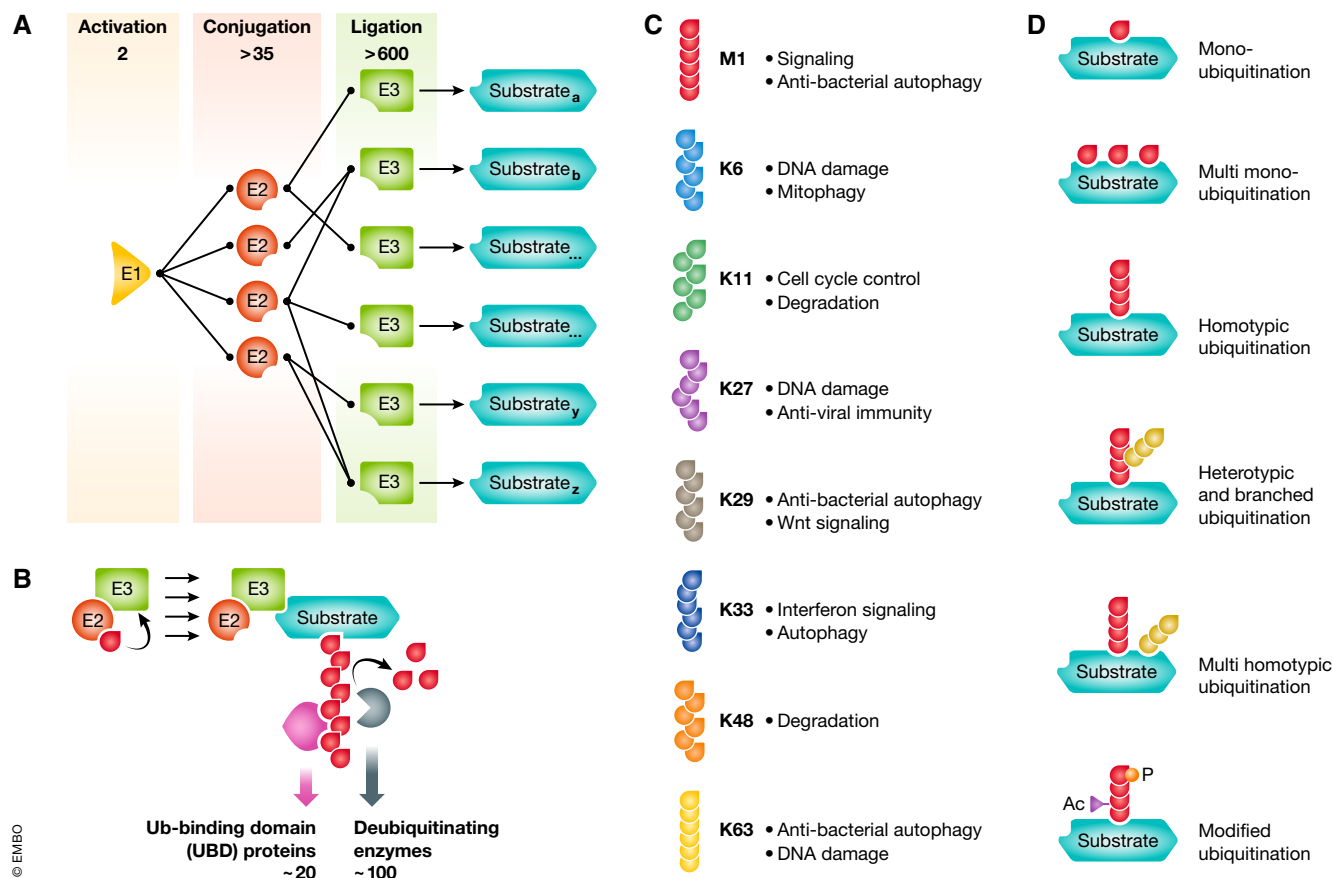
Figure 1. The complexity of ubiquitin conjugation.

(A) Schematic representation of the abundance and interactions of human ubiquitin-activating enzyme (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin protein ligases (E3s) involved in ubiquitination. (B) E3 ubiquitin-protein ligases (like for example RING E3s) recruit ubiquitin-loaded E2 enzymes and substrates and mediate the formation of ubiquitin chains. These chains can be recognized by ubiquitin-binding domain (UBD) proteins and/or degraded by deubiquitinating enzymes in a chain-selective manner. (C) The repertoire of ubiquitin chains, linked through methionine (M) 1 (linear/head-to-tail) or through the internal lysine (K) residues 6, 11, 27, 29, 33, 48 and 63 with a short description of their cellular function. (D) Overview of several modes of substrate ubiquitination including different forms of mono- and polyubiquitination and the post-translational modification of ubiquitin itself by acetylation (Ac) and phosphorylation (P).

Methods to study ubiquitination

Ever since its discovery, biochemistry- and imaging-based approaches to study ubiquitination are continuously evolving, improving and adapting. In recent years, ubiquitin biochemistry has profited from the identification of novel key enzymes, new heterotypic/branched chain types and novel pathways relying on ubiquitin. Imaging-based approaches are now more and more complementing biochemical methods due to novel developments and applications in reagents to visualize ubiquitin chains with confocal and super-resolution microscopy.

Biochemical approaches to study ubiquitination

Ubiquitination is classically studied by resolving ubiquitin chains and/or ubiquitinated substrates on Western blot, and biochemical experiments are the method of choice for substrate identification. Antibody-/affinity reagent-based substrate/chain enrichment allows

mass spectrometry to further discover novel aspects of ubiquitination. Since these methods have been extensively reviewed elsewhere (see, for example, the reviews of [25] and [26]), here we only want to highlight some of the most useful tools that were developed in recent years. In particular, the antibody-based enrichment of Ub Gly-Gly-Lysine substrate peptides upon trypsinization ((diGly) ubiquitin remnant proteomics) has enabled powerful and versatile substrate identification in complex biological specimens [26]. Furthermore, the development of tandem ubiquitin-binding entities (TUBEs), in which one or multiple UBDs are fused, has proven to be powerful for chain enrichment and substrate identification [27–29]. Since then, application-specific TUBE adjustments have been introduced, such as ubiquitin chain protection from trypsinization (Ub-ProT) to determine ubiquitin chain length [30] and sensor-based chain-specific TUBEs, like the linear ubiquitin-specific M1-specific ubiquitin binder (SUB) [31]. Interestingly, TUBE-like chain-binding sensors are used in cellular imaging-based experiments as well (discussed in more detail later). Genetic trapping approaches like ubiquitin ligase trapping [32,33] and ubiquitin-activated interaction

Box 1:   Ubiquitin mutants and derivatives for microscopic analysis of cellular ubiquitination
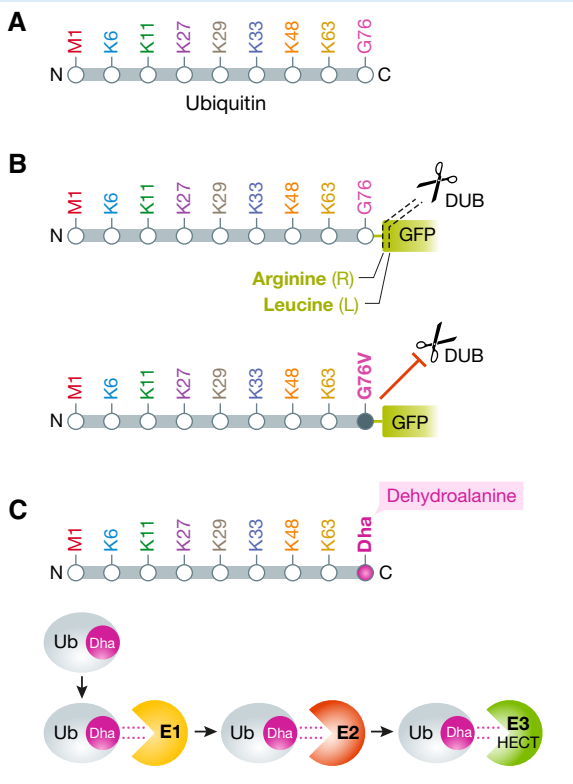
Schematic representation of the ubiquitin molecule. (A) Depicted are the N- and C-termini, the initiator methionine (M1) for linear ubiquitination, the seven internal lysine residues and the C-terminal glycine-76. (B) Two exemplary ubiquitin-green fluorescent protein (GFP) fusion protein reporters, used to image ubiquitin/proteasome-dependent proteolysis and the degradative functions of ubiquitin. DUB-mediated cleavage of ubiquitin-(R)-GFP or ubiquitin-(L)-GFP give rise to GFP molecules with arginine or leucine at the N-terminus that determine the half-lives of the GFP molecules by the N-end rule pathway (*upper*). The deubiquitinating enzyme (DUB)-resistant ubiquitin G76V mutant becomes modified with ubiquitin chains that mediate subsequent proteasomal degradation of the reporter, leading to a decrease in GFP signals (*lower*). (C) Ubiquitin derivatives with dehydroalanine (Dha) at position 76 can be used as cascade probes to investigate the cellular paths of ubiquitin, including the E1, E2 and HECT E3 ligase (see text for info).

traps (UBAITs) further facilitated substrate identification [34]. Additionally, orthogonal Ub transfer (OUT) relies on the expression of engineered E1, E2 and E3 enzymes (xE1, xE2 and xE3) that possess reactivity towards an affinity tagged ubiquitin mutant (xUb), but not to endogenous Ub, leading to the identification of selective substrates [35,36]. The applicability of this elegant approach has been demonstrated successfully by the identification of novel E6AP and CHIP E3 ligase substrates [37,38]. Although these approaches have not been adapted to microscopy-based settings yet, it should theoretically be possible to image substrate ubiquitination with known, tagged E3 ligase and substrate pairs and labelled ubiquitin. Finally, chemical biology, combined with structural information,

has yielded a wealth of activity-based probes that can be used to manipulate and study key enzymes in ubiquitin research (see for an overview for example [39–41]). Chemical and semi-chemical probes have been developed that target E1, E2 and E3 enzymes and E3-substrate interactions [42–44], DUBs [45–48], UBDs [49] or can be applied for the induction of protein degradation, such as proteolysis-targeting chimeras (PROTACs) [50–54].

In conclusion, biochemistry-based methods are ideally suited for substrate identification, the verification of chain specificity, the differentiation between mono- and polyubiquitination and can be applied on a wide range of biological materials, ranging from *in vitro* ubiquitination reactions, cellular lysates to whole tissues and organisms. However, biochemical measurements often occur post-lysis and can potentially increase the incidence of artefacts. Moreover, protein interactions might be too weak to be detected by immunoprecipitation and Western blotting. Furthermore, restriction of Ub reactions to specific cellular compartments or subsets of targets often require cell fractionation to enrich specific substrates or chain types. Scaling-up to high-throughput or high-content settings is also difficult to achieve and provides limited spatial-temporal resolution (Table 1).

Strategies to monitor ubiquitination in mammalian cells using microscopy

Complementing the abovementioned approaches, recent developments in optical microscopy have opened the door to image, visualize and trace ubiquitin-related processes directly in native and live-cell settings. In particular, the development of microscopy techniques that achieve a spatial resolution approaching the size of single proteins allows functional studies on how proteins organize and interact at the molecular level in their physiological environment. In the following paragraphs, we highlight three important strategies to image cellular ubiquitination and discuss specific advantages and disadvantages. The first approach utilizes fluorescently labelled reporter and model substrates to indirectly image the degradative functions of ubiquitination and the UPS. The second approach applies tagged ubiquitin reagents, chain-specific antibodies and chain-specific sensors to directly image ubiquitination in cellular compartments and biological processes. Finally, the application of super-resolution microscopy allows to image ubiquitin signals in mammalian cells with unprecedented spatial resolution.

Indirect imaging of ubiquitination in protein degradation
The role of ubiquitination in proteasomal degradation has been extensively studied by imaging the stability and degradation of artificial reporter proteins and physiological model substrates. Certain ubiquitin signals like ubiquitin chains linked through K11 and K48 serve as recognition signals for degradation by the 26S proteasome [2,17,55]. Fluorescently labelled, degradation-sensitive reporters have been developed, often based on green fluorescent protein (GFP) or derivatives, which are stabilized or degraded when expressed in isolated cells or intact organisms (Fig 2A) [56,57]. Monitoring changes in fluorescence intensity serves as indirect read-out for ubiquitination and proteasome function and has for example facilitated the development and evaluation of proteasome inhibiting compounds [56,57].

Table 1. Comparative advantages and disadvantages of biochemistry- and imaging-based approaches to study cellular aspects of ubiquitination

	Advantages	Disadvantages
Biochemistry-based	• Method of choice for substrate identification • Chain-specific • Differentiation between mono- and polyubiquitination • Qualitative/quantitative applications • Applicable on tissues and intact organisms	• Cell lysis required • Cell fractionation often required • Quantitative (near) single-molecule experiments are difficult • High-throughput/high-content analysis difficult • Often time-consuming • Limited spatial resolution • Limited temporal resolution • Often specialized (and expensive) measurement/analysis set-ups needed
Imaging-based	• Real-time live-cell imaging possible • Chain-specific • Native environment • No cell fractionation required, use of organelle markers • Quantitative single-molecule imaging possible • Qualitative/quantitative applications • High-throughput/high-content analysis possible • Applicable on tissues and intact organisms • High spatial resolution • High temporal resolution	• Not well suitable for substrate identification • Potential fixation and permeabilization artefacts • Limited differentiation between mono- and polyubiquitination • Often specialized (and expensive) imaging set-ups needed

One of the first GFP-based UPS reporter (GFPu) was generated by fusing GFP to the 16-residue CL1 degron that becomes degraded by the 26S proteasome in an ubiquitin-dependent manner [58,59]. This GFP-based reporter has been applied to indirectly study UPS function in isolated cells and intact organisms and can be used to investigate protein degradation in specific cellular compartments, like cytosol and the nucleus [60]. Another reporter that probes the activity of DUBs consists of a direct fusion of ubiquitin to GFP. DUB-dependent cleavage of the ubiquitin molecule generates free GFP with different N-terminal residues that serve as indicators for N-end rule pathways degradation since the N-terminal residue determines the protein half-live [61,62]. In addition, the non-cleavable UbG76V-GFP and –Dendra2 fusion reporters become modified with polyubiquitin chains that subsequently target the complete fusion protein for 26S proteasomal degradation (Fig 2A) and (Box 1) [61,63,64]. These reporters have been applied in isolated cells and transgenic UbG76V-GFP reporter mice to study proteasome activity [64].

The above listed reporters either employ artificial substrates or indirectly monitor DUB and proteasome activity. An alternative approach is the direct fluorescent labelling of the physiological ubiquitin substrate itself. This strategy has been applied to NF-κB signalling (Fig 2B). Under basal conditions, the transcription factor p65/RelA is retained in the cytoplasm by its interaction with IκBα. Pathway activation induces IKK-dependent IκBα phosphorylation and its ubiquitin-dependent degradation leading to the liberation and nuclear translocation of p65 [65]. The co-imaging of the nucleo-cytoplasmic shuttling of GFP-labelled p65 with the oscillatory accumulation/degradation of mCherry-tagged IκBα protein has allowed to draw conclusions on NF-κB kinetics, dynamics and oscillations [66–68].

Another prominent example is the cell cycle indicator FUCCI (fluorescence ubiquitination cell cycle indicator). Here, two proteins known to be degraded in specific phases of the cell cycle are tagged with two different fluorescent reporters: RFP-Cdt1 and GFP-Geminin (Fig 2C) [69]. During S, G2 and M phases of the cell cycle, RFP-Cdt

is degraded by the UPS, giving rise to GFP-positive nuclei, whereas GFP-tagged geminin is degraded in G1 with the red signal remaining. The G1/S transition phase shows yellow fluorescent nuclei, due to the green and red overlay, since Cdt1 levels are decreasing and geminin levels increase [69]. A variant of FUCCI, called FUCCI4, combines four different fluorescent substrates (mKO2-Cdt (30–120), mTurquoise2-SLBP (18–126), Clover-Geminin (1–110) and H1.0-Maroon) to allow visualization of each cell cycle phase [70]. In addition, Fly-FUCCI has been developed to monitor proliferation in tissues, based on the FUCCI principle [71]. The FUCCI principle allows thus a dynamic, indirect imaging of the degradative functions of ubiquitination during cell cycle progression and division.

An interesting development is the application of nanobodies. These are single-chain V_HH antibody regions specifically designed to recognize a specific epitope. These small, monomeric and stable reagents, derived from immunized *Camelidae* sp., can be labelled with fluorophores and expressed in cells [72,73]. Expression of these so-called chromobodies can be achieved by conventional transient expression methods or through stable integration using viral transduction in a constitutive or inducible manner, depending on experimental constraints. Chromobodies against a wide variety of endogenous epitopes [72,73] including GFP are available. GFP nanobodies can have GFP-quenching or GFP-stimulating properties [74] and can be genetically tagged to be used as biochemical matrices to enrich GFP-tagged proteins [74]. In an elegant approach, GFP nanobodies have been employed to achieve the selective (ubiquitin-dependent) degradation of any GFP-tagged protein of interest [75]. The authors fused the *Drosophila* F-box protein Slmb to GFP nanobodies (NSlmb-vhhGFP4). Since F-box proteins are the substrate specifying determinants of large multimeric E3 ligase complexes, called SKP1-CUL1-F-Box (SCF) complexes [76], recognition of GFP-tagged proteins by NSlmb-vhhGFP4 induces their degradation by the SCF complex. The authors demonstrated the applicability of deGradFP (degrade green fluorescent protein) in
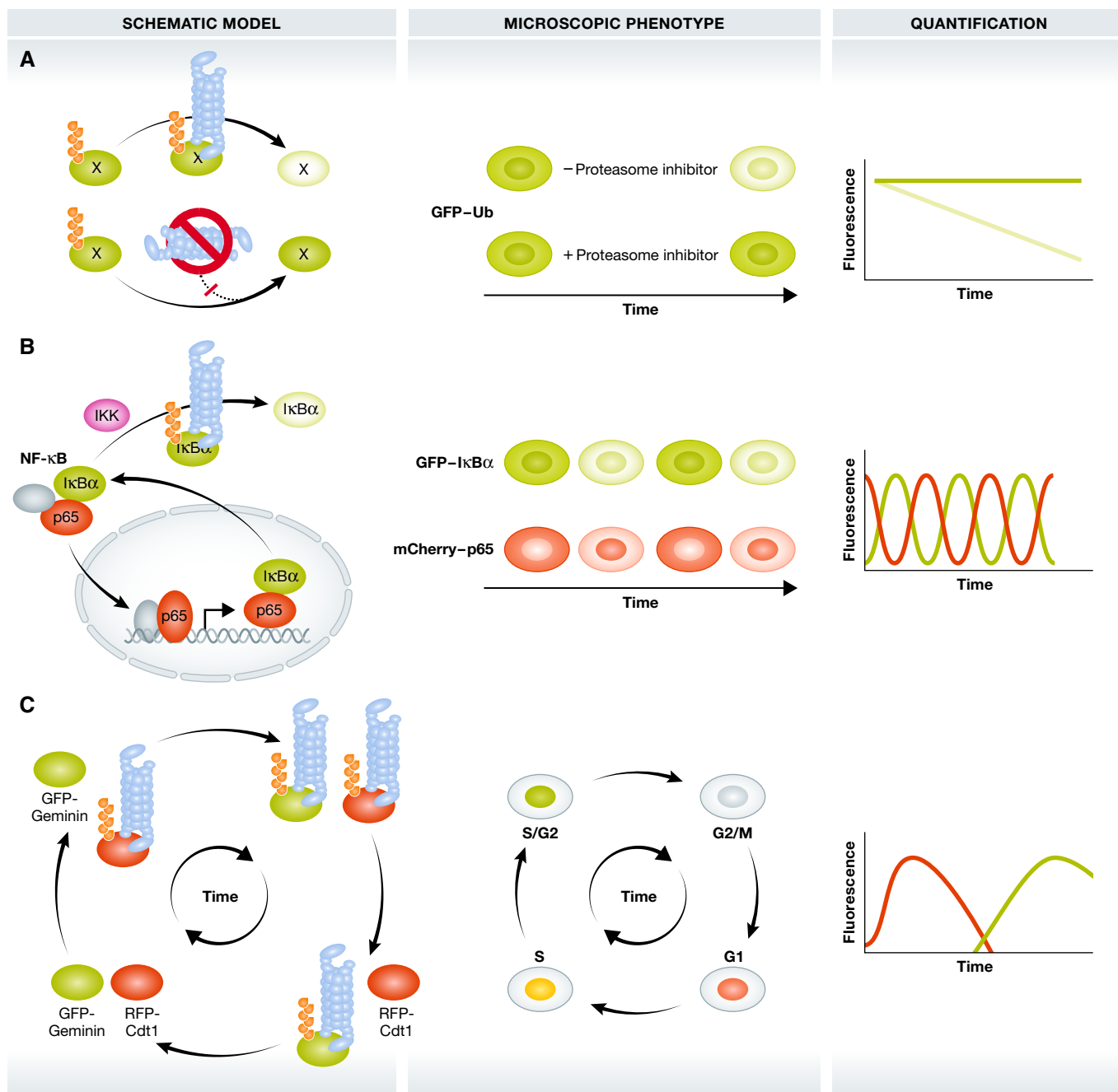
Figure 2. Indirect imaging of the degradative functions of ubiquitin.
Approaches to image the proteasome-related functions of Ub in the control of protein stability and breakdown. Darker and lighter green colours indicate the accumulation and breakdown of proteins, respectively. (A) GFP-labelled Ub or model substrates are modified with degradative Ub signals and degraded by the 26S proteasome (blue barrel). Proteasome inhibition induces stabilization and accumulation of these GFP reporters as ubiquitinated forms. (B) Upon IKK activation, GFP-tagged IκBα becomes modified with K48-linked polyubiquitin chains and degraded by the 26S proteasome. This releases mCherry-RelA/p65 that subsequently translocates in the nucleus to control NF-κB-dependent gene expression. IκBα is among these NF-κB target genes and shuttles back into the cytosol, creating dynamic NF-κB degradation/translocation loops. Grey ovals: additional NF-κB transcription factors (C) FUCCI: co-expression of the UPS substrates GFP-Geminin and RFP-Cdt1 allows microscopic analysis of cell cycle phases by phase-dependent Ub-dependent degradation of GFP-Geminin and RFP-Cdt1.

isolated cells and intact organisms [75]. It would be interesting to apply this GFP knockout technique on the degradation of GFP-tagged ubiquitin chain-specific sensors proteins, like GFP-UBAN, that selectively bind linear Ub chains. Theoretically, one would expect proteasomal degradation of the GFP chain sensors and perhaps of the endogenous linear Ub chains as well. This would imply novel modes of manipulation of ubiquitin signalling that can easily be combined with imaging-based experiments.

Table 2. Useful reagents for monitoring cellular ubiquitination using microscopy

Application	Reagent	Advantages	Disadvantages
Degradative proteasomal functions of Ub in biological processes	GFP-tagged wild-type ubiquitin and mutants	Robust and applicable in intact animals	Transfection required Potential influence on Ub homeostasis Limited to degradative functions of the ubiquitin/proteasome system
	GFP-tagged physiological Ub substrates	Pathways often characterized in detail	Transfection required Limited to degradative functions of the ubiquitin/proteasome system
	Degron-tagged nanobodies against GFP	Target flexibility	Transfection required Limited to degradative functions of the ubiquitin/proteasome system
Visualization of Ub chains, substrates and processes in cellular structures and processes	Ectopic expression of tagged Ub	Powerful in combination with biochemistry Ubiquitin mutants for chain characterization	Transfection required Competition with endogenous Ub Potential influence on Ub homeostasis Tags at Ub might interfere with function Ectopic expression of Ub mutants might influence native chain patterns N-terminal tagging interferes with linear chain formation
	Ub chain sensors	Chain selective Applicable in living cells Compatible with BiFC Potential influences of sensors of process of interest can be used to discover novel chain functions	Transfection required Prone to influencing process of interest by interfering with endogenous chain recognition Prone to high background signals Target binding potentially susceptible to post-translational modification of Ub chains
	Ub antibodies	Easy to use in immunofluorescence Characterized in detail Compatible with library screening and affinity maturation and optimization	Post-fixation Only available against selected Ub chain targets
	Ub affimers	Versatile, especially in combination with library screening Small and physiochemically robust Compatible with genetic tagging and ectopic expression	Post-fixation
	Ub(Dha) derivatives	Promising reagents for imaging Ub flux Compatible with live-cell imaging and in principle with FRAP/FRET	Electroporation required Optimization required

In conclusion, several reporters, methods and reagents have been developed and applied that are useful for studying Ub and protein breakdown by the 26S proteasome in imaging-based set-ups. Although these techniques do not allow direct imaging of the degradative Ub signals, the focus is on imaging the functional consequences of these signals on reporter stability. 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. These reporter-based read-outs are less suitable for monitoring proteasome-independent functions of ubiquitin and do not answer questions about the specific type of ubiquitin modification.

Direct imaging of ubiquitination in fixed and living cells

Microscopy-based imaging of ubiquitination in cells is mostly focused on the visualization of local accumulations of different ubiquitin signals. In contrast to diffuse ubiquitination reactions, that might take place freely in certain organelles, accumulated ubiquitinated structures, like aggregates, foci or puncta, provide assemblies that can be imaged easily. These structures are in most cases ensembles of mixed types of ubiquitin chains, likely combined with

(multiple) mono-ubiquitination and/or branched chains. Two main approaches are currently in use to image ubiquitin, a direct one in which genetically labelled ubiquitin is used and an indirect one using reagents that recognize certain types of ubiquitin signals (Table 2).

The most straightforward way of direct imaging of ubiquitination includes the transient or stable overexpression of tagged or fluorescent labelled ubiquitin in mammalian cells [77]. These ubiquitin molecules are recognized by the E1-E2-E3 machinery and incorporated into chains. Direct visualization of GFP or immunofluorescence with antibodies against specific tag proteins allows selective imaging. Although this method seems straightforward, a few points have to be considered. First, the ectopically expressed ubiquitin has to compete with the endogenous ubiquitin molecules for incorporation, so sufficient levels of (over)expression need to be achieved. However, ectopic expression of ubiquitin on itself might already affect cellular processes and the (de)ubiquitination balance. Second, it cannot be excluded that tags, especially bulky ones, like GFP, interfere with chain growth and accessibility by UBD proteins. Third, expression of labelled wild-type ubiquitin does not provide information about the type of ubiquitin chain. To solve this,
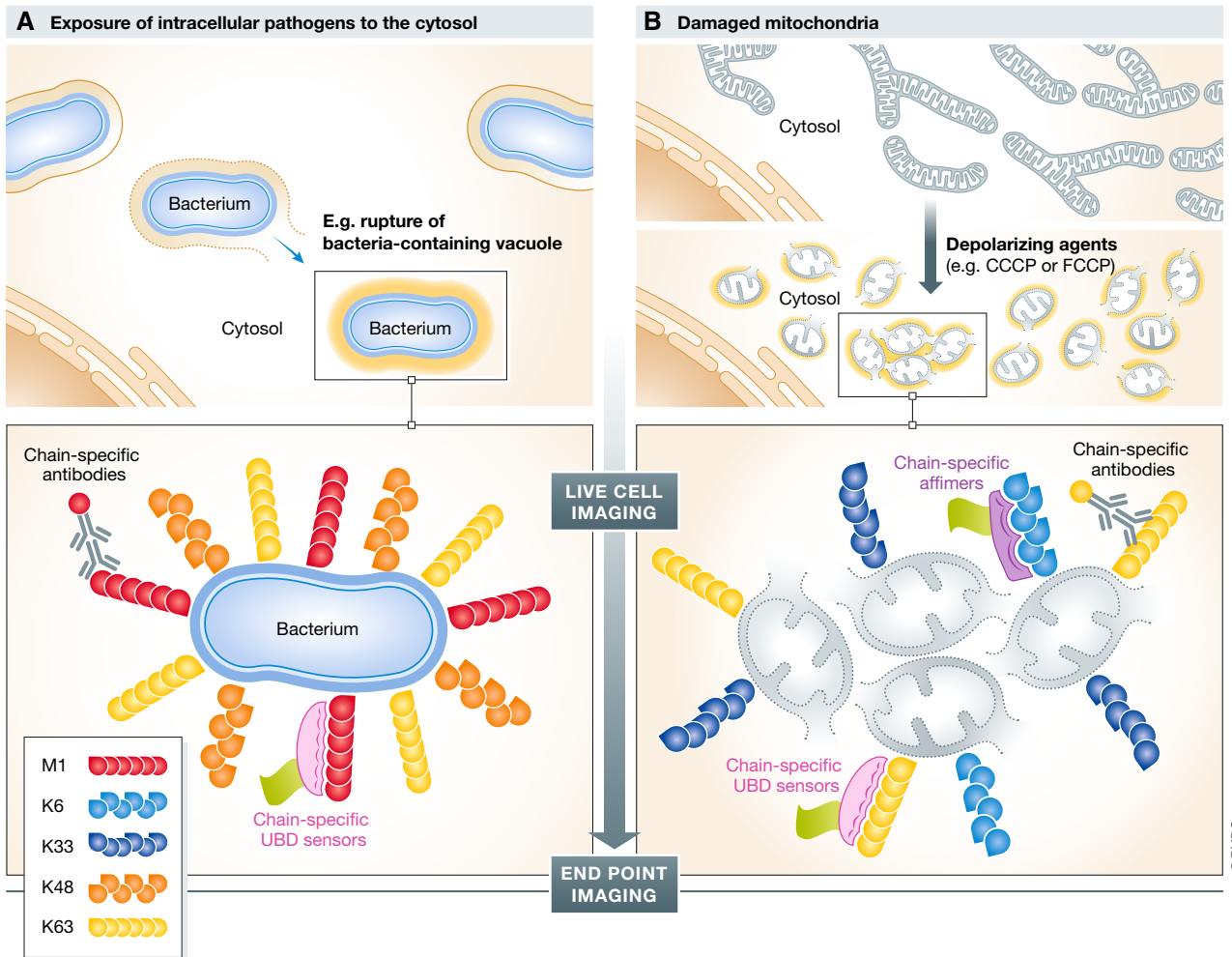
Figure 3. Imaging ubiquitination in selective autophagy.
Schematic overview of live-cell and end-point Ub imaging applications in antibacterial autophagy (xenophagy) and autophagic breakdown of damaged mitochondria (mitophagy). (A) Intracellular pathogens, like *Salmonella*, reside mostly in *Salmonella*-containing vacuoles. In some cases, vacuolar membrane rupture and pathogens are exposed to the host cytosol, inducing prominent multi-type Ub deposition at the bacterial surface. These Ub structures serve as non-self "eat me" signals that trigger bacterial autophagy. (B) Damaged mitochondria *(grey circles)*, for example induced by depolarizing agents like CCCP/FCCP, trigger PINK1/PARKIN-dependent ubiquitination at the surface of these organelles, thereby recruiting the autophagy machinery. In both examples, Ub chain-specific antibodies, UBD-based chain-specific sensors and chain-specific affimers were applied to understand the mechanisms and functional relevance of ubiquitination. Substantial overlap in similarities of Ub patterns exists in these two mechanistically distinct forms of ubiquitin-accumulated structures and the contribution of Ub for selective autophagy.

lysine-to-arginine mutation of the internal residues within ubiquitin can be used, but one needs to be cautious with interpreting the obtained results since the mutations might force the generation of specific chains and require sufficient levels of expression in order to function adequately [77]. Finally, placing a tag at the N-terminus of ubiquitin interferes with the ability to form linear chains [78]. A strategy to overcome this negative effect of tagging the N-terminus of ubiquitin is the recent development of lysine-less, internally STREP II-tagged ubiquitin (INT-Ub.7KR) [78]. INT-Ub.7KR can be used as mono-Ub or as part of linear Ub chains, but INT-Ub.7KR is expected to be incorporated in virtually every chain type as terminal ubiquitin, so care must be taken concerning this compromised specificity. Up to now, INT-Ub.7KR has only been applied in mass spectrometry, but internally tagged Ub should allow imaging ubiquitination with microscopy techniques.

Apart from tracing ubiquitin mutants and variants, expression of isolated, chain-specific UBDs fused to GFP enable visualization and tracking of specific ubiquitin chains in cells (Fig 3). Several UBD-based sensors have been generated and applied. Among the first is the GFP-labelled, triple fused ubiquitin-interacting motif (UIM) domain of VPS27 that specifically monitors K63-linked chains *in vitro* and in cells [79]. Other sensors use a single GFP-tagged UBAN domain of NEMO to visualize M1 ubiquitination or the TAB2 Npl4 zinc finger (NZF) domain to detect K63-linked chains [80]. These entities allowed imaging of Ub signals in DNA damage responses, mitochondrial damage and pathogen invasion in fixed and living cells (Fig 3A and B) [79,80]. The current repertoire of UBD-based sensors is limited to linear, K33, K48 and K63 chains. Recently, a global chain-interaction screen using immobilized, non-hydrolysable di-Ub molecules covering the complete spectrum of
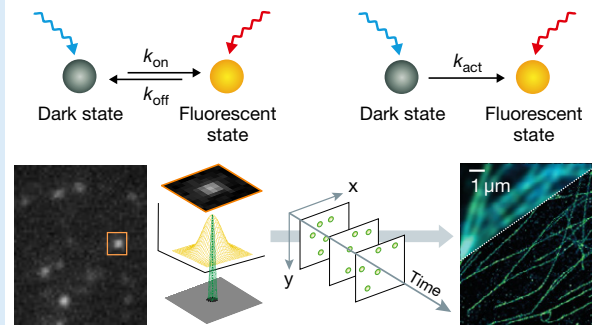
homotypic linkages was performed using ubiquitin interactor affinity enrichment-mass spectrometry (UbIA-MS) [81]. This screen identified many putative interactors for Ub chains, like UCHL3 for K27 chains and ACTL8 as K6 interactor that could be further developed into chain-specific sensors. Bimolecular fluorescence complementation (BiFC) represents an interesting alternative to the application of single or tandem fused UBDs. BiFC is based on the reconstitution of fluorescence of two non-fluorescent fragments of a normally fluorescent protein, like Venus, a yellow fluorescent protein (YFP) variant [82]. When proteins fused to these fragments come in close proximity, the two inert halves reconstitute a functional fluorescent protein [82]. Ubiquitination-induced fluorescence complementation (UiFC) is a variant of BiFC that has been used to image K48-linked chains in cells. Three tandem K48-specific UIM domains of epsin1 fused to the N- and C-terminal fragments of Venus [83] indeed detected K48-linked polyubiquitin chains *in vitro* and in fixed and living cells. The principle has been applied to image K48 ubiquitin chain dynamics in presynaptic assembly and differentiation [83,84]. In addition, direct fusion of certain ubiquitin mutants to two split fragments of the Kusabira-Green (KG) fluorescent protein generated a platform (polyubiquitin-mediated fluorescence complementation, polyUb-FC) that allowed the imaging of K33 polyubiquitination chains and linked K33 chains to autophagy signalling [85]. The specific advantage of this approach is that background fluorescence is strongly reduced, since the Kusabira-Green protein only emits when the two Kusabira fragments are incorporated into a chain, i.e. as intact fluorescent protein.

Although ectopically expressed UBD-based sensors have been useful in monitoring cellular ubiquitination in fixed and living cells, there are several aspects that need to be taken into account. First, due to their selective binding capacities, often combined with high affinities, sensors may act as interfering reagents that hinder access of endogenous UBD proteins and DUBs. This may lead to artificial stabilization of sensor-bound chains or disturbances in Ub-based signalling, especially concerning chain types that are less abundant. Furthermore, post-translational modification of ubiquitin in chains and/or branched chain types might interfere with sensor recognition and binding. Finally, introduction of sensor molecules requires transfection procedures, which need to be optimized for individual cell types. On the other hand, these inhibitory characteristics can also be employed to resolve the contribution of certain ubiquitin linkages to biological processes, such as the significance of M1/K63-linked chains for TNFR1-mediated NF-κB and interleukin signalling [79,80]. Ideally, the delivery of chain-specific sensors should be done using inducible expression systems and careful sensor titrations combined with phenotypic assessments.
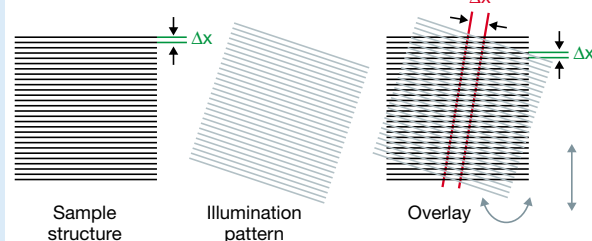
Besides the abovementioned approaches that rely on the ectopic expression of modified forms of ubiquitin or UBDs, general and chain-specific ubiquitin antibodies have been developed that can be used for immunofluorescence [86–90]. Although the majority of these antibodies were originally used for enrichment procedures using immunoprecipitation, some of these, like for example FK1 and FK2, do efficiently recognize ubiquitinated structures in fixed and permeabilized cells prepared for fluorescence microscopy [86,91]. The monoclonal antibody FK1 recognizes polyubiquitinated proteins but not monoubiquitinated or free ubiquitin. FK2 on the other hand recognizes both mono- and polyubiquitinated substrates [86,91]. Care must be taken since the binding properties of these antibodies have been validated using Western blotting with purified and

isolated ubiquitin reagents and might depend on experimental settings, like immobilization of the antibody. Indeed, it has been shown recently that the FK2 monoclonal antibody exhibits some preference for certain linkages, at least in recognizing the eight types of di-ubiquitin on Western blot [92].

Box 2:   Three concepts of optical super-resolution microscopy
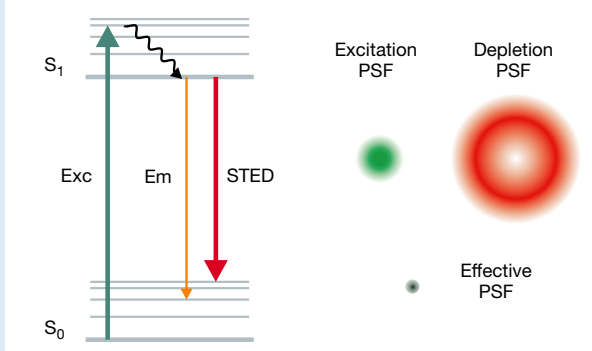
Three concepts of optical super-resolution microscopy. In single-molecule localization microscopy (SMLM), an image is generated by determining the precise position of single fluorophore labels. This requires a separation of fluorophores during imaging, which is either achieved by photoswitching/photoactivation, or by fluorophore labels that transiently bind to a target. In structured illumination microscopy (SIM), a sample is illuminated with a periodic pattern, generating moiré fringes. From various translations and rotations of this pattern, a set of images is recorded, and a high-resolution image is reconstructed computationally. Stimulated emission depletion (STED) employs targeted switching of fluorescence achieved by overlaying an excitation laser with a doughnut-shaped (null intensity in the centre) de-excitation beam, generating a sub-diffraction sized, effective excitation spot. Figures were adapted from [153] and [154].

To specifically detect linkage-specific ubiquitin chains, phage display technologies combined with binding optimization have generated antibodies that selectively recognize linear, K11, K48, K63 and a bispecific antibody recognizing K11/K48 heterotypic chains [17,87–90]. These antibodies have been applied successfully in immunoprecipitation experiments as well as confocal fluorescence and super-resolution microscopy (see below) [89].

Affimers are novel and alternative chain-specific reagents, often based on a 12-kDa cystatin fold scaffold that contains an alpha-helix and an anti-parallel beta-sheet connected through loop regions [93]. These loop regions can be randomized and screened from phage display libraries to bind a protein of interest with high affinity and specificity [93]. Affimers are generally much smaller as antibodies or isolated UBDs and are pH stable and thermally robust and allow versatile affimer applications as well as chemical and genetic modification and enable imaging experiments [94,95]. Ub chain-specific affimers have been developed by screening of affimer variant libraries for binders of K6 and K33/K11 linkages [96,97]. Structure-based optimization including dimerization improved the performance of these ubiquitin chain affimers and increased target chain affinities [97]. K6-specific affimers were used to study mechanistic aspects of K6 chain generation in a broad array of biochemical and cell biological approaches and led to the identification of HUWE1 as K6-specific E3 ligase in cells [97]. In addition, chemical labelling of K6 affimers with AlexaFluor488 allowed the detection of K6-linked polyubiquitinated structures on damaged mitochondria and identified USP30 as DUB for K6-ubiquitinated TOM20 by fluorescence microscopy (Fig 3B) [97]. A major advantage of affimers as chain-specific antibody-like reagents compared to overexpression other ubiquitin-binding entities is the lack of confounding inhibitory effects due to ectopic expression. At present affimers are not expressed in cells, but are being used as antibodies after specimen fixation. However, intracellular expression of chain-specific affimers could potentially be used to understand the biological function of uncommon ubiquitin chains by competing for chain access with endogenous UBDs and/or DUBs. Recently, affimers against SUMO1 and SUMO2/3 have been described as well, highlighting the potential of this technology for other types of post-translation modifications [98].

A promising contribution towards cellular imaging of Ub functions comes from a remarkable type of activity-based probe, called UbDHA, that has been developed as synthetic cascading probe that enables monitoring of sequential E1, E2 and HECT-type E3 ligase activity [43] (Box 1). Inspired by the observation that the G76A Ub variant still can be used by the E1-E2-E3 machinery, although with decreased efficiency [99,100], UbDHA is composed of residues 1–75 of Ub G76A in which the C-terminal alanine is replaced by dehydroalanine (Dha). Upon activation of UbDha by the E1 enzyme, Dha can either be covalently linked to the E1 enzyme in a E1-UbDha-thioether adduct or follow the native ligation pathway to E2 and E3 enzymes. At the level of E2 enzymes, UbDha can again form E2-thioether adducts or travel further to active site cysteine E3 ligases, i.e. HECT and RBR-type E3s. This probe has been validated thoroughly in vitro and in vivo [43]. Intriguingly, Cy5-labelled UbDha has been introduced in cells by electroporation together with ectopic expression of GFP-tagged UBE1, the main mammalian E1 enzyme. Upon co-expression with GFP-UBE1, Cy5-UbDha was found enriched within the nucleus, colocalizing with GFP-UBE1. Furthermore, Cy5-UbDha colocalized with the wild-type GFP-UBE2J2 E2 enzyme, but not with the catalytically inactive C91S mutant. In addition, this colocalization was dependent on the upstream E1, since E1 inhibition with PYR-41 inhibited colocalization. The imaging-based experiments of the UbDha probe highlight the possibilities of applying activity-based probes for ubiquitination to measure ubiquitin "flux" in cells. Introducing these probes in living cells would allow Fluorescence Recovery After Photobleaching (FRAP)-like experiments (see below). For this, probe fluorescence could be bleached in certain cellular regions of interest and the redistribution of fluorescence could be monitored in time and space and provide information on Ub dynamics in cells. Combining this analysis with measuring colocalization of specific UPS components, like GFP-UBE1, this approach should enable measurement of UPS flux. At the moment, Cy5-UbDha imaging is limited to co-imaging the probe with GFP-labelled UPS enzymes and more insights concerning the UbDha transfer efficiencies in cells would be needed. It would be extremely interesting to further test and develop probes to image ubiquitination in cells. However, one potential disadvantage of chemical probes is that these entities are introduced into cells using electroporation that potentially might influence ubiquitination and signalling cascades.

The dynamic interchange of ubiquitin has been studied using FRAP in combination with GFP- and photoactivatable GFP (paGFP)-labelled Ub in different cellular compartments and with proteasome inhibition [101], disease-associated protein aggregates [102,103] and autophagy [104]. Furthermore, Förster Resonance Energy Transfer (FRET) has been applied for studying example DUB selectivity [105,106] and regulation of E3 function [107], although mostly applied in in vitro applications.

In conclusion, the continuous development of new and innovative reagents to image ubiquitination has clearly facilitated ubiquitin research. The application of structure-guided optimization of new

Figure 4. Super-resolution imaging-based differentiation of novel roles for differentially linked polyUb in bacterial autophagy.
(A) Schematic depiction of dSTORM-based imaging using chain-specific Ub antibodies to analyse the role of differential ubiquitination on the surface of cytosolic *Salmonella*. Ubiquitinated cytosolic *Salmonella* (blue) labelled with Ub chain-specific antibodies against M1 (*red chains*) and K63 (*yellow chains*) combined with secondary antibodies carrying dSTORM-compatible fluorescent labels (*red and green dots*). Locating single, antibody-associated fluorophores allows reconstruction of super-resolution images and mathematical modelling of the fluorophore distribution and accumulation. (B) dSTORM imaging of the nanoscale distribution of total ubiquitin and chain-specific ubiquitin patterns around cytosolic *Salmonella* in human epithelial cells reveals the importance of the linear deubiquitinating enzyme OTULIN for controlling the distribution of linear ubiquitin patterns at ubiquitin-coated intracellular bacteria (reproduced with permission in adapted form from [138]). (C) dSTORM SRM imaging-based quantification detected an increased density and altered M1 distribution patterns on cytosolic *Salmonella* upon loss of the DUB OTULIN. This could indicate (I) modification of novel substrates Y and Z with M1 chains by LUBAC that are normally deubiquitinated by OTULIN, (II) more M1 chains on native substrate X that are removed in the presence of OTULIN or (III) longer M1 chains on native substrate X that are normally trimmed by OTULIN. (D) The higher spatial resolution of dSTORM and SIM imaging compared to confocal laser scanning microscopy (CLSM) reveals the spatial organization of different ubiquitin chains on the surface of *Salmonella*. NEMO is recruited to M1 chains at the bacterial surface resulting in the accumulation of phosphorylated IKK and the activation of NF-κB pro-survival signalling. See text for more details.
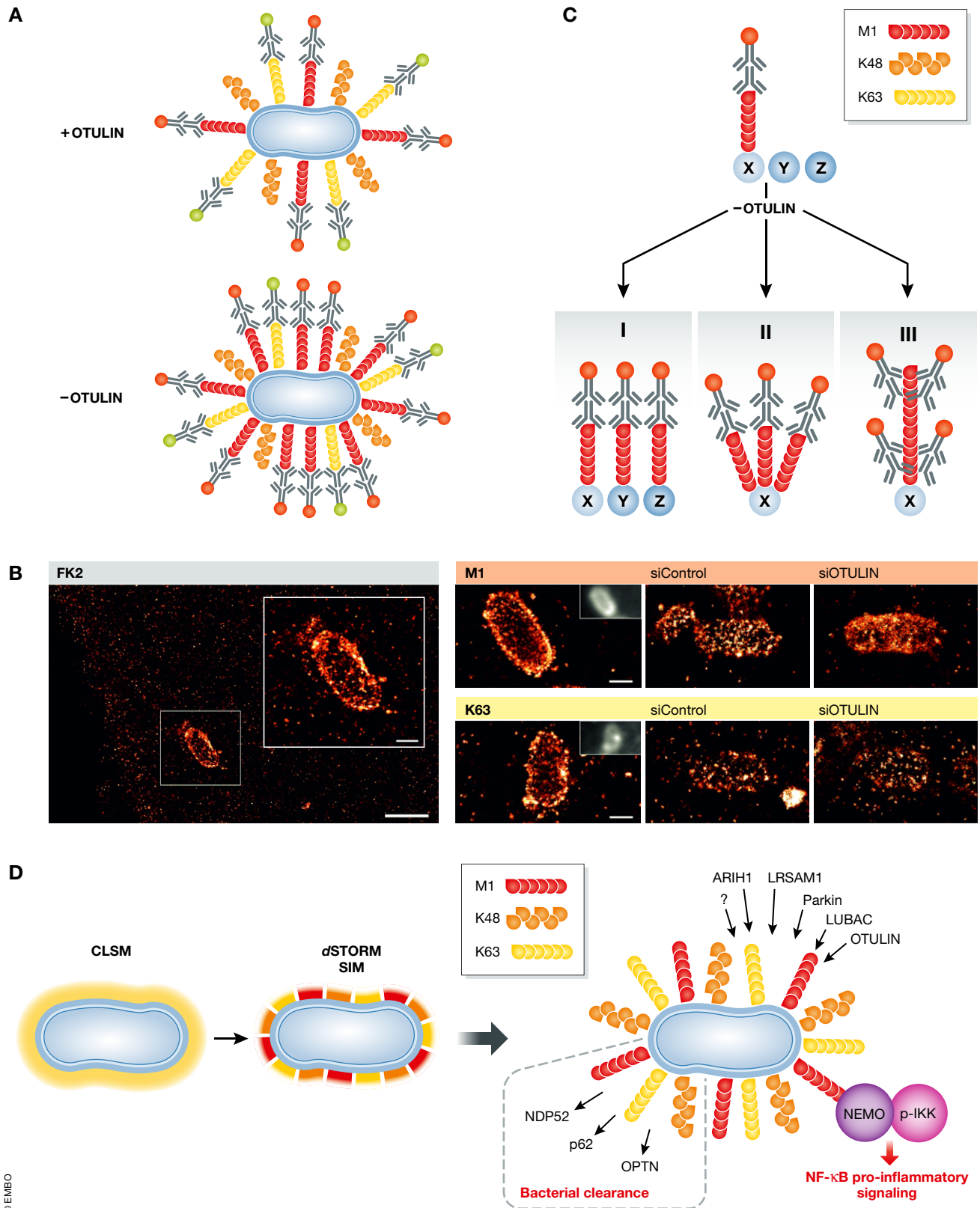
Figure 4.

methodologies is expected to open new avenues that will further increase our understanding of ubiquitination in cell biology. One of the major remaining problems is that current conventional ways to image ubiquitination focus on imaging dense and accumulated ubiquitinated structures within cells. In other words, current techniques image larger ensembles of ubiquitin signals, including multiple substrates, often modified on multiple residues, modified with multiple types of homo- and heterotypic ubiquitin chains of different lengths and different stoichiometry. Diffraction-limited optical microscopy provides insufficient spatial resolution to uncover molecular details concerning ubiquitination in cells, and therefore, the application of super-resolution microscopy to study ubiquitination might be a promising approach to overcome these problems [108].

Super-resolution imaging of ubiquitination

Super-resolution microscopy (SRM) methods can provide unprecedented levels of spatial resolution into the distribution of biological molecules and processes, such as the molecular architecture of protein complexes, membrane nanostructures and protein aggregates [108]. Although the majority of ubiquitin imaging applications use conventional fluorescence microscopy, ectopic expression of labelled ubiquitin and antibody staining of ubiquitin chains is ideally suitable for SRM. While the spatial resolution in conventional fluorescence microscopy is restricted to about 200 nm laterally and 500 nm axially, common SRM techniques reach a spatial resolution of 10–20 nm, thus approaching the size of proteins. In addition, single-molecule super-resolution methods can provide quantitative information on protein copy numbers and protein complex stoichiometry. The repertoire of super-resolution methodologies, technologies and applications is growing and was comprehensively reviewed before [108–110].

At present, SRM methods that have been successfully used to image ubiquitination are patterned light illuminated techniques, such as structured illumination microscopy (SIM) [111] and localization-based technologies, like direct stochastic optical reconstruction microscopy (*d*STORM) [112] and photoactivated localization microscopy (PALM) [113] (Box 2). Apart from these, stimulated emission depletion (STED) microscopy [114] and (lattice) light-sheet microscopy [115] can in principle also be used for ubiquitin SRM imaging. SIM uses a finely stripped, patterned illumination (structured illumination) to image a sample, which will generate interference patterns (moiré patterns). These patterns are coarser than the original and can be detected by optical microscopes. SIM captures these patterns with slightly varying the orientation of the structured illumination and processes these images to mathematically reconstruct a high-resolution structure of the specimen. *d*STORM and PALM are examples of single-molecule localization microscopy (SMLM) and rely on sequential activation and localization of only a subset of fluorophores within a sample. The exact fluorophore position can be determined by finding the centre of the point-spread function of individual fluorophores. By iterative cycles of activation and deactivation, a super-resolution image can be constructed [116]. SMLM can be realized with organic fluorophores or with photoconvertible fluorescent proteins. An alternative strategy to achieve labelling of a subset of targets is to use reversible duplex formation of DNA, as in point accumulation for imaging in nanoscale topography (DNA-PAINT) [117,118]. SMLM has been applied for three-dimensional

imaging, for example by using optical astigmatism in which an elliptical shape of the point-spread function (PSF) provides information concerning the x-y-z directions of the fluorophores [119].

Multi-wavelength super-resolution imaging of ubiquitination as well as SRM of living cells is still challenging. As for all microscopy-based approaches to study ubiquitination, SRM is dependent on the level of fluorescence intensity compared to background to allow proper image acquisition and detection. Ubiquitin antibodies are generally working well for immunofluorescence, but if the ubiquitin structures of interest are less abundant, adequate detection might be hampered. This becomes especially valid when there is insufficient contrast between the region of interest and adjacent structures. Therefore, SRM-based techniques are ideally suited for imaging ubiquitination at accumulated structures, like aggregates, organelles or invading pathogens. This is illustrated by 3D SIM imaging of the SUMO 2/3 distribution at arsenic-induced PML nuclear bodies that provide information on how PML becomes degraded in an ubiquitin- and SUMO-dependent manner [120]. Another example is the application of single-molecule localization microscopy to study the localization of E3 ligases, like Smurf2, STUB1/CHIP, Cullin RING E3 ligase with the KLHL21 adaptor protein (CRL3^KLHL21), proteasome storage granules in yeast and proteins closely colocalizing with general and chain-specific ubiquitin signals [121–125]. Therefore, dense structures and ubiquitination occurring at the surfaces of these structures serve as excellent platforms to image ubiquitin homeostasis and to understand the functions of E3 ligases and DUBs.

Imaging ubiquitination in bacterial autophagy and pro-inflammatory signalling

The surface of intracellular bacteria, such as *Salmonella*, is a prototypic example of such dense structures and provides an ideal model system to image many aspects of ubiquitination. Exposure to pathogenic bacteria requires host defence strategies to restrict bacterial proliferation and spread. *Salmonella enterica* subs. *enterica* Typhimurium (hereafter referred to as *Salmonella*) invade host cells by inducing rearrangements in the actin network and the cytoskeleton [126]. Most intracellular bacteria reside and replicate in membrane-derived, dedicated *Salmonella*-containing vacuoles (SCVs) that provide shielding against host immune surveillances [126]. Due to the nutritional status of the mammalian cytosol, certain subpopulations of vacuolar *Salmonella* escape and translocate into the cytoplasm [127]. These bacteria are covered with dense structures of ubiquitin [127–129]. *Salmonella* Ub coats act as potent stimulator of bacterial clearance through selective autophagy by recruiting autophagy receptors like SQSTM1/p62, CALCOCO2/NDP52 and optineurin (OPTN) [130–132]. These proteins mediate the formation of double-membraned autophagosomes that target bacteria for lysosomal destruction in a process called xenophagy [129].

Ubiquitinated intracellular bacteria provide excellent models to image ubiquitin homeostasis for several reasons. First, bacteria are rather large and can be very easily imaged with confocal fluorescence microscopy [133]. Second, the onset of ubiquitination is timed and only occurs when bacteria are exposed to the host cytosol, which is relatively well described for *Salmonella* [128]. Third, antibodies against bacterial cell wall markers and immunofluorescent markers for the autophagic machinery are available that allow

co-staining with fluorescent bacteria. Fourth, multiple types of ubiquitin chains can be found in the *Salmonella* ubiquitin coat. For example, immunofluorescence staining of *Salmonella*-infected human epithelial cells with FK1, FK2 and chain-specific antibodies and imaging of the recruitment of GFP-tagged linear and K63-specific UBDs expressed in infected cells confirmed the presence of linear, K11-, K63- and K48-linked chains around the cytosolic *Salmonella* [80,92,128,133–135]. These approaches contributed to the identification and characterization of key E3 ligases, like LUBAC [92], PARKIN [135], LRSAM1 [136] and ARIH1 [137], of the DUB OTULIN [138] and several UBD proteins that recruit the autophagy machinery (like SQSTM1/p62, NDP52 and OPTN) [130–132,139,140]. In addition, multiscale imaging has been applied to explore the role of Ub in xenophagy of other intracellular microorganisms, like *Shigella* and *Mycobacterium* [135,141] and even internalized latex beads that mimic cytosolic bacteria [142].

Recently, SIM and *d*STORM imaging was performed on cytosolic *Salmonella* total, M1, K63 and K48 ubiquitin and revealed unprecedented levels of resolution [92,138]. SRM confirmed non-uniform distributions of several of these ubiquitin structures within the coat in which some chain-linkages accumulated in micro domain-like structures [92,138]. In addition, SIM was also used to image the distribution of the autophagic adaptor proteins p62 and NDP52 on the type II *Toxoplasma gondii* parasitophorous vacuole [140]. Quantitative *d*STORM imaging of M1 signals in the Salmonella Ub coat revealed striking alterations in M1 antibody distribution and intensity in infected OTULIN-depleted cells, without affecting K63 Ub, suggesting OTULIN as crucial regulator of linear chain deposition within the ubiquitin coat (Fig 4A and B) [138]. These quantitative measurements highlight the potential of applying SRM in monitoring ubiquitination in cells. At present, SRM cannot discriminate between changes in M1 chain lengths or the number of M1 chains within the bacterial Ub surface upon loss of OTULIN expression. Therefore, changes in M1 antibody intensity could indicate OTULIN-dependent increases in the length of linear chains or M1 modification of novel substrates by LUBAC (Fig 4C).

Modulating M1 homeostasis by altering LUBAC or OTULIN levels allowed visualizing of the balance between linear ubiquitin conjugation and deconjugation in cells [92,138]. Apart from mediating bacterial autophagy, increased M1 Ub also recruits NEMO, an M1-specific UBD with well-described roles in TNF-mediated NF-κB signalling [143], and the IKK complex [143–146]. Imaging IKK activation by quantifying IKK serine 176 and serine 180 phosphorylation using immunofluorescence at the bacterial Ub coat indicate local activation of canonical NF-κB activation directly on the bacterial Ub surface (Fig 4D) [92,138].

Taken together, these observations emphasize the usefulness and relevance of Ub CSLM and SRM to discover novel parallels between antibacterial autophagy and pro-inflammatory signalling in host cell autonomous signalling. Further application and combination of Ub imaging with existing biochemical methodologies is expected to identify novel roles of ubiquitination at different levels of resolution.

Conclusions

In recent years, advances in microscopic methodologies to study ubiquitination contributed immensely to our understanding of the

In need of answers

Although many basic aspects of cellular ubiquitination have been elucidated in recent years, some central questions in the ubiquitin research field remain unanswered.

(i)   Development of labelling and microscopic tools to image the complete "ubiquitin code" with molecular resolution in cellular settings, especially:
   a. Robust and versatile methods for real-time analysis of chain formation and/or breakdown using sensors and FRET/FRAP analysis would be extremely useful in the analysis of certain E3/DUBs on cellular ubiquitination reactions.
   b. Versatile chemical probes to specifically monitor the ubiquitin flux over E1, E2, E3 enzymes, DUBs or structural compartments would also contribute to our understanding of cellular ubiquitination events, especially those that can be applied in (live-cell) imaging-based applications.
(ii) What are the spatial molecular patterns of E3 ligases and DUBs and how is ubiquitin chain distribution and length controlled at the molecular level in a cell?
(iii) How is ubiquitin and substrate degradation organized by the 26S proteasome in cells at the molecular level?
(iv) How is the ubiquitin exchange and flux regulated on ubiquitinated surfaces of (dense) cellular structures?
(v)   What are the molecular mechanisms of diffuse ubiquitination reactions in not well-defined compartments?

cellular roles of ubiquitin. Application and improvements of microscopic Ub imaging in a wide variety of physiologically relevant scenarios have increased our insights in this fundamental type of post-translational modification. Conventional fluorescence microscopy and super-resolution imaging emerge as valuable supplements for existing biochemical and mass-spectrometric methodologies and open novel possibilities to image ubiquitination in real time in living cells. Multi-disciplinary approaches to monitor ubiquitination will open new areas of ubiquitin research, especially quantitative cellular aspects with single-molecule resolution. Ongoing developments in biochemical reagents and mass spectrometry-assisted ubiquitinome profiling, CRISPR/Cas9 and label-free proteomics already allowed to map ubiquitination sites and substrates in an organismal setting with unprecedented depth. Finding ways to combine reagents that specifically label Ub chains, based on recombinant affimers and chain-selective binding moieties, is expected to further reveal important insights in cellular Ub homeostasis and functions.

Cellular imaging with super-resolution will increase our understanding of functional aspects of ubiquitination. A broad repertoire of probes, UBD sensors, affimers and antibodies are available and compatible with various super-resolution techniques. In order to answer fundamental questions in the ubiquitin research field, such as measuring chain length in cells or visualize substrate modification in native environments, quantitative methods will be beneficial, such as single-molecule counting approaches [147–149] and advanced biophysical techniques, like fluorescence (cross) correlation spectroscopy (FC(C)S) [150]. For live-cell studies, novel approaches that employ exchangeable fluorophores allow for long-term super-resolution imaging with minimal photobleaching [151,152].

Imaging ubiquitination with increased spatial resolution will unravel novel quantitative aspects of ubiquitination and ubiquitin chains in mammalian cells and permits quantitative measurements of ubiquitin chain parameters and associated protein networks in

the future. Finally, current Ub imaging focus on larger ubiquitinated cellular structures, like protein aggregates, organelles and intracellular pathogens as ideal reaction platforms to study ubiquitin homeostasis, chain conformation and E3/DUB selectivity in cells. Application of multi-level ubiquitin imaging approaches in structurally not well-defined cellular compartments, like membranes or diffuse cytosolic ubiquitination reactions, will be a major future challenge for ubiquitin research.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

References

1.　Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67: 425–479

2.　Dikic I (2017) Proteasomal and autophagic degradation systems. *Annu Rev Biochem* 86: 193–224

3.　Behrends C, Harper JW (2011) Constructing and decoding unconventional ubiquitin chains. *Nat Struct Mol Biol* 18: 520–528

4.　Rape M (2017) Ubiquitylation at the crossroads of development and disease. *Nat Rev Mol Cell Biol*, 19: 59–70

5.　Mahajan R, Delphin C, Guan T, Gerace L, Melchior F (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88: 97–107

6.　Matunis MJ, Coutavas E, Blobel G (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* 135: 1457–1470

7.　Loeb KR, Haas AL (1992) The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. *J Biol Chem* 267: 7806–7813

8.　Kamitani T, Kito K, Nguyen HP, Yeh ET (1997) Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. *J Biol Chem* 272: 28557–28562

9.　Pearce MJ, Mintseris J, Ferreyra J, Gygi SP, Darwin KH (2008) Ubiquitin-like protein involved in the proteasome pathway of Mycobacterium tuberculosis. *Science* 322: 1104–1107

10.　Lehmann G, Udasin RG, Livneh I, Ciechanover A (2017) Identification of UBact, a ubiquitin-like protein, along with other homologous components of a conjugation system and the proteasome in different gram-negative bacteria. *Biochem Biophys Res Commun* 483: 946–950

11.　Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* 286: 309–312

12.　Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, Pavletich NP (1999) Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* 286: 1321–1326

13.　Eisenhaber B, Chumak N, Eisenhaber F, Hauser MT (2007) The ring between ring fingers (RBR) protein family. *Genome Biol* 8: 209

14.　Schulman BA (2011) Twists and turns in ubiquitin-like protein conjugation cascades. *Protein Sci* 20: 1941–1954

15.　Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K, Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J* 25: 4877–4887

16.　Ikeda F, Dikic I (2008) Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: beyond the Usual Suspects' review series. *EMBO Rep* 9: 536–542

17.　Yau RG, Doerner K, Castellanos ER, Haakonsen DL, Werner A, Wang N, Yang XW, Martinez-Martin N, Matsumoto ML, Dixit VM *et al* (2017) Assembly and function of heterotypic ubiquitin chains in cell-cycle and protein quality control. *Cell* 171: 918–933.e920

18.　Emmerich CH, Ordureau A, Strickson S, Arthur JS, Pedrioli PG, Komander D, Cohen P (2013) Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc Natl Acad Sci USA* 110: 15247–15252

19.　Trempe JF (2011) Reading the ubiquitin postal code. *Curr Opin Struct Biol* 21: 792–801

20.　Husnjak K, Dikic I (2012) Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu Rev Biochem* 81: 291–322

21.　Rahighi S, Dikic I (2012) Selectivity of the ubiquitin-binding modules. *FEBS Lett* 586: 2705–2710

22.　Randles L, Walters KJ (2012) Ubiquitin and its binding domains. *Front Biosci* 17: 2140–2157

23.　Mevissen TET, Komander D (2017) Mechanisms of deubiquitinase specificity and regulation. *Annu Rev Biochem* 86: 159–192

24.　Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123: 773–786

25.　Iconomou M, Saunders DN (2016) Systematic approaches to identify E3 ligase substrates. *Biochem J* 473: 4083–4101

26.　Ordureau A, Munch C, Harper JW (2015) Quantifying ubiquitin signaling. *Mol Cell* 58: 660–676

27.　Yoshida Y, Saeki Y, Murakami A, Kawawaki J, Tsuchiya H, Yoshihara H, Shindo M, Tanaka K (2015) A comprehensive method for detecting ubiquitinated substrates using TR-TUBE. *Proc Natl Acad Sci USA* 112: 4630–4635

28.　Hjerpe R, Aillet F, Lopitz-Otsoa F, Lang V, England P, Rodriguez MS (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep* 10: 1250–1258

29.　Emmerich CH, Cohen P (2015) Optimising methods for the preservation, capture and identification of ubiquitin chains and ubiquitylated proteins by immunoblotting. *Biochem Biophys Res Commun* 466: 1–14

30.　Tsuchiya H, Burana D, Ohtake F, Arai N, Kaiho A, Komada M, Tanaka K, Saeki Y (2018) Ub-ProT reveals global length and composition of protein ubiquitylation in cells. *Nat Commun* 9: 524

31.　Keusekotten K, Elliott PR, Glockner L, Fiil BK, Damgaard RB, Kulathu Y, Wauer T, Hospenthal MK, Gyrd-Hansen M, Krappmann D *et al* (2013) OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell* 153: 1312–1326

32.　Mark KG, Simonetta M, Maiolica A, Seller CA, Toczyski DP (2014) Ubiquitin ligase trapping identifies an SCF(Saf1) pathway targeting unprocessed vacuolar/lysosomal proteins. *Mol Cell* 53: 148–161

33.　Mark KG, Loveless TB, Toczyski DP (2016) Isolation of ubiquitinated substrates by tandem affinity purification of E3 ligase-polyubiquitin-binding domain fusions (ligase traps). *Nat Protoc* 11: 291–301

34. O'Connor HF, Lyon N, Leung JW, Agarwal P, Swaim CD, Miller KM, Huibregtse JM (2015) Ubiquitin-activated interaction traps (UBAITs) identify E3 ligase binding partners. *EMBO Rep* 16: 1699–1712

35. Zhao B, Bhuripanyo K, Zhang K, Kiyokawa H, Schindelin H, Yin J (2012) Orthogonal ubiquitin transfer through engineered E1-E2 cascades for protein ubiquitination. *Chem Biol* 19: 1265–1277

36. Liu X, Zhao B, Sun L, Bhuripanyo K, Wang Y, Bi Y, Davuluri RV, Duong DM, Nanavati D, Yin J *et al* (2017) Orthogonal ubiquitin transfer identifies ubiquitination substrates under differential control by the two ubiquitin activating enzymes. *Nat Commun* 8: 14286

37. Wang Y, Liu X, Zhou L, Duong D, Bhuripanyo K, Zhao B, Zhou H, Liu R, Bi Y, Kiyokawa H *et al* (2017) Identifying the ubiquitination targets of E6AP by orthogonal ubiquitin transfer. *Nat Commun* 8: 2232

38. Bhuripanyo K, Wang Y, Liu X, Zhou L, Liu R, Duong D, Zhao B, Bi Y, Zhou H, Chen G *et al* (2018) Identifying the substrate proteins of U-box E3s E4B and CHIP by orthogonal ubiquitin transfer. *Sci Adv* 4: e1701393

39. Witting KF, Mulder MPC, Ovaa H (2017) Advancing our understanding of ubiquitination using the Ub-Toolkit. *J Mol Biol* 429: 3388–3394

40. Ovaa H, Vertegaal ACO (2018) Probing ubiquitin and SUMO conjugation and deconjugation. *Biochem Soc Trans* 46: 423–436

41. Hewings DS, Flygare JA, Bogyo M, Wertz IE (2017) Activity-based probes for the ubiquitin conjugation-deconjugation machinery: new chemistries, new tools, and new insights. *FEBS J* 284: 1555–1576

42. Scott DC, Hammill JT, Min J, Rhee DY, Connelly M, Sviderskiy VO, Bhasin D, Chen Y, Ong SS, Chai SC *et al* (2017) Blocking an N-terminal acetylation-dependent protein interaction inhibits an E3 ligase. *Nat Chem Biol* 13: 850–857

43. Mulder MP, Witting K, Berlin I, Pruneda JN, Wu KP, Chang JG, Merkx R, Bialas J, Groettrup M, Vertegaal AC *et al* (2016) A cascading activity-based probe sequentially targets E1-E2-E3 ubiquitin enzymes. *Nat Chem Biol* 12: 523–530

44. Pao KC, Stanley M, Han C, Lai YC, Murphy P, Balk K, Wood NT, Corti O, Corvol JC, Muqit MM *et al* (2016) Probes of ubiquitin E3 ligases enable systematic dissection of parkin activation. *Nat Chem Biol* 12: 324–331

45. Weber A, Elliott PR, Pinto-Fernandez A, Bonham S, Kessler BM, Komander D, El Oualid F, Krappmann D (2017) A linear diubiquitin-based probe for efficient and selective detection of the deubiquitinating enzyme OTULIN. *Cell Chem Biol* 24: 1299–1313.e1297

46. Flierman D, van der Heden van Noort GJ, Ekkebus R, Geurink PP, Mevissen TE, Hospenthal MK, Komander D, Ovaa H (2016) Non-hydrolyzable diubiquitin probes reveal linkage-specific reactivity of deubiquitylating enzymes mediated by S2 pockets. *Cell Chem Biol* 23: 472–482

47. Tan XD, Pan M, Gao S, Zheng Y, Shi J, Li YM (2017) A diubiquitin-based photoaffinity probe for profiling K27-linkage targeting deubiquitinases. *Chem Commun (Camb)* 53: 10208–10211

48. de Jong A, Merkx R, Berlin I, Rodenko B, Wijdeven RH, El Atmioui D, Yalcin Z, Robson CN, Neefjes JJ, Ovaa H (2012) Ubiquitin-based probes prepared by total synthesis to profile the activity of deubiquitinating enzymes. *ChemBioChem* 13: 2251–2258

49. Liang J, Zhang L, Tan XL, Qi YK, Feng S, Deng H, Yan Y, Zheng JS, Liu L, Tian CL (2017) Chemical synthesis of diubiquitin-based photoaffinity probes for selectively profiling ubiquitin-binding proteins. *Angew Chem Int Ed Engl* 56: 2744–2748

50. Uehara T, Minoshima Y, Sagane K, Sugi NH, Mitsuhashi KO, Yamamoto N, Kamiyama H, Takahashi K, Kotake Y, Uesugi M *et al* (2017) Selective degradation of splicing factor CAPERalpha by anticancer sulfonamides. *Nat Chem Biol* 13: 675–680

51. Mot AC, Prell E, Klecker M, Naumann C, Faden F, Westermann B, Dissmeyer N (2018) Real-time detection of N-end rule-mediated ubiquitination via fluorescently labeled substrate probes. *New Phytol* 217: 613–624

52. Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, Dhe-Paganon S, Bradner JE (2015) Drug Development. Phthalimide conjugation as a strategy for *in vivo* target protein degradation. *Science* 348: 1376–1381

53. Sakamoto KM, Kim KB, Kumagai A, Mercurio F, Crews CM, Deshaies RJ (2001) Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc Natl Acad Sci USA* 98: 8554–8559

54. Bondeson DP, Mares A, Smith IE, Ko E, Campos S, Miah AH, Mulholland KE, Routly N, Buckley DL, Gustafson JL *et al* (2015) Catalytic *in vivo* protein knockdown by small-molecule PROTACs. *Nat Chem Biol* 11: 611–617

55. Meyer HJ, Rape M (2014) Enhanced protein degradation by branched ubiquitin chains. *Cell* 157: 910–921

56. Matilainen O, Jha S, Holmberg CI (2016) Fluorescent tools for *in vivo* studies on the ubiquitin-proteasome system. *Methods Mol Biol* 1449: 215–222

57. Menendez-Benito V, Heessen S, Dantuma NP (2005) Monitoring of ubiquitin-dependent proteolysis with green fluorescent protein substrates. *Methods Enzymol* 399: 490–511

58. Gilon T, Chomsky O, Kulka RG (1998) Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. *EMBO J* 17: 2759–2766

59. Bence NF, Sampat RM, Kopito RR (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 292: 1552–1555

60. Bence NF, Bennett EJ, Kopito RR (2005) Application and analysis of the GFPu family of ubiquitin-proteasome system reporters. *Methods Enzymol* 399: 481–490

61. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* 18: 538–543

62. Bachmair A, Finley D, Varshavsky A (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* 234: 179–186

63. Stack JH, Whitney M, Rodems SM, Pollok BA (2000) A ubiquitin-based tagging system for controlled modulation of protein stability. *Nat Biotechnol* 18: 1298–1302

64. Hamer G, Matilainen O, Holmberg CI (2010) A photoconvertible reporter of the ubiquitin-proteasome system *in vivo*. *Nat Methods* 7: 473–478

65. Baeuerle PA, Baltimore D (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242: 540–546

66. Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG *et al* (2004) Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* 306: 704–708

67. Nelson G, Paraoan L, Spiller DG, Wilde GJ, Browne MA, Djali PK, Unitt JF, Sullivan E, Floettmann E, White MR (2002) Multi-parameter analysis of the kinetics of NF-kappaB signalling and transcription in single living cells. *J Cell Sci* 115: 1137–1148

68. Kovalenko A, Wallach D (2006) If the prophet does not come to the mountain: dynamics of signaling complexes in NF-kappaB activation. *Mol Cell* 22: 433–436

69. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H *et al* (2008) Visualizing

spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132: 487–498

70. Bajar BT, Lam AJ, Badiee RK, Oh YH, Chu J, Zhou XX, Kim N, Kim BB, Chung M, Yablonovitch AL *et al* (2016) Fluorescent indicators for simultaneous reporting of all four cell cycle phases. *Nat Methods* 13: 993–996

71. Zielke N, Korzelius J, van Straaten M, Bender K, Schuhknecht GF, Dutta D, Xiang J, Edgar BA (2014) Fly-FUCCI: a versatile tool for studying cell proliferation in complex tissues. *Cell Rep* 7: 588–598

72. Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, Leonhardt H (2008) A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol Cell Proteomics* 7: 282–289

73. Rothbauer U, Zolghadr K, Tillib S, Nowak D, Schermelleh L, Gahl A, Backmann N, Conrath K, Muyldermans S, Cardoso MC *et al* (2006) Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods* 3: 887–889

74. Kirchhofer A, Helma J, Schmidthals K, Frauer C, Cui S, Karcher A, Pellis M, Muyldermans S, Casas-Delucchi CS, Cardoso MC *et al* (2010) Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol* 17: 133–138

75. Caussinus E, Kanca O, Affolter M (2011) Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat Struct Mol Biol* 19: 117–121

76. Skaar JR, Pagan JK, Pagano M (2013) Mechanisms and function of substrate recruitment by F-box proteins. *Nat Rev Mol Cell Biol* 14: 369–381

77. Volk S, Wang M, Pickart CM (2005) Chemical and genetic strategies for manipulating polyubiquitin chain structure. *Methods Enzymol* 399: 3–20

78. Kliza K, Taumer C, Pinzuti I, Franz-Wachtel M, Kunzelmann S, Stieglitz B, Macek B, Husnjak K (2017) Internally tagged ubiquitin: a tool to identify linear polyubiquitin-modified proteins by mass spectrometry. *Nat Methods* 14: 504–512

79. Sims JJ, Scavone F, Cooper EM, Kane LA, Youle RJ, Boeke JD, Cohen RE (2012) Polyubiquitin-sensor proteins reveal localization and linkage-type dependence of cellular ubiquitin signaling. *Nat Methods* 9: 303–309

80. van Wijk SJ, Fiskin E, Putyrski M, Pampaloni F, Hou J, Wild P, Kensche T, Grecco HE, Bastiaens P, Dikic I (2012) Fluorescence-based sensors to monitor localization and functions of linear and K63-linked ubiquitin chains in cells. *Mol Cell* 47: 797–809

81. Zhang X, Smits AH, van Tilburg GB, Jansen PW, Makowski MM, Ovaa H, Vermeulen M (2017) An interaction landscape of ubiquitin signaling. *Mol Cell* 65: 941–955.e948

82. Kerppola TK (2009) Visualization of molecular interactions using bimolecular fluorescence complementation analysis: characteristics of protein fragment complementation. *Chem Soc Rev* 38: 2876–2886

83. Chen Z, Zhong Y, Wang Y, Xu S, Liu Z, Baskakov IV, Monteiro MJ, Karbowski M, Shen Y, Fang S (2013) Ubiquitination-induced fluorescence complementation (UiFC) for detection of K48 ubiquitin chains *in vitro* and in live cells. *PLoS One* 8: e73482

84. Pinto MJ, Pedro JR, Costa RO, Almeida RD (2016) Visualizing K48 ubiquitination during presynaptic formation by ubiquitination-induced fluorescence complementation (UiFC). *Front Mol Neurosci* 9: 43

85. Nibe Y, Oshima S, Kobayashi M, Maeyashiki C, Matsuzawa Y, Otsubo K, Matsuda H, Aonuma E, Nemoto Y, Nagaishi T *et al* (2018) Novel polyubiquitin imaging system, PolyUb-FC, reveals that K33-linked polyubiquitin is recruited by SQSTM1/p62. *Autophagy*, 14: 347–358

86. Fujimuro M, Yokosawa H (2005) Production of antipolyubiquitin monoclonal antibodies and their use for characterization and isolation of polyubiquitinated proteins. *Methods Enzymol* 399: 75–86

87. Matsumoto ML, Dong KC, Yu C, Phu L, Gao X, Hannoush RN, Hymowitz SG, Kirkpatrick DS, Dixit VM, Kelley RF (2012) Engineering and structural characterization of a linear polyubiquitin-specific antibody. *J Mol Biol* 418: 134–144

88. Matsumoto ML, Wickliffe KE, Dong KC, Yu C, Bosanac I, Bustos D, Phu L, Kirkpatrick DS, Hymowitz SG, Rape M *et al* (2010) K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol Cell* 39: 477–484

89. Newton K, Matsumoto ML, Ferrando RE, Wickliffe KE, Rape M, Kelley RF, Dixit VM (2012) Using linkage-specific monoclonal antibodies to analyze cellular ubiquitylation. *Methods Mol Biol* 832: 185–196

90. Newton K, Matsumoto ML, Wertz IE, Kirkpatrick DS, Lill JR, Tan J, Dugger D, Gordon N, Sidhu SS, Fellouse FA *et al* (2008) Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell* 134: 668–678

91. Fujimuro M, Sawada H, Yokosawa H (1994) Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett* 349: 173–180

92. Noad J, von der Malsburg A, Pathe C, Michel MA, Komander D, Randow F (2017) LUBAC-synthesized linear ubiquitin chains restrict cytosol-invading bacteria by activating autophagy and NF-kappaB. *Nat Microbiol* 2: 17063

93. Tiede C, Bedford R, Heseltine SJ, Smith G, Wijetunga I, Ross R, AlQallaf D, Roberts AP, Balls A, Curd A *et al* (2017) Affimer proteins are versatile and renewable affinity reagents. *Elife* 6: e24903

94. Schlichthaerle T, Eklund AS, Schueder F, Strauss MT, Tiede C, Curd A, Ries J, Peckham M, Tomlinson DC, Jungmann R (2018) Site-specific labeling of affimers for DNA-PAINT microscopy. *Angew Chem Int Ed Engl*, 57: 11060–11063

95. Bedford R, Tiede C, Hughes R, Curd A, McPherson MJ, Peckham M, Tomlinson DC (2017) Alternative reagents to antibodies in imaging applications. *Biophys Rev* 9: 299–308

96. Gersch M, Gladkova C, Schubert AF, Michel MA, Maslen S, Komander D (2017) Mechanism and regulation of the Lys6-selective deubiquitinase USP30. *Nat Struct Mol Biol* 24: 920–930

97. Michel MA, Swatek KN, Hospenthal MK, Komander D (2017) Ubiquitin linkage-specific affimers reveal insights into k6-linked ubiquitin signaling. *Mol Cell* 68: 233–246.e235

98. Hughes DJ, Tiede C, Penswick N, Tang AA, Trinh CH, Mandal U, Zajac KZ, Gaule T, Howell G, Edwards TA *et al* (2017) Generation of specific inhibitors of SUMO-1- and SUMO-2/3-mediated protein-protein interactions using Affimer (Adhiron) technology. *Sci Signal* 10: eaaj2005

99. Hodgins RR, Ellison KS, Ellison MJ (1992) Expression of a ubiquitin derivative that conjugates to protein irreversibly produces phenotypes consistent with a ubiquitin deficiency. *J Biol Chem* 267: 8807–8812

100. Pickart CM, Kasperek EM, Beal R, Kim A (1994) Substrate properties of site-specific mutant ubiquitin protein (G76A) reveal unexpected mechanistic features of ubiquitin-activating enzyme (E1). *J Biol Chem* 269: 7115–7123

101. Dantuma NP, Groothuis TA, Salomons FA, Neefjes J (2006) A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling. *J Cell Biol* 173: 19–26

102. Ben Yehuda A, Risheq M, Novoplansky O, Bersuker K, Kopito RR, Goldberg M, Brandeis M (2017) Ubiquitin accumulation on disease

associated protein aggregates is correlated with nuclear ubiquitin depletion, histone De-ubiquitination and impaired DNA damage response. *PLoS One* 12: e0169054

103. Juenemann K, Jansen AHP, van Riel L, Merkx R, Mulder MPC, An H, Statsyuk A, Kirstein J, Ovaa H, Reits EA (2018) Dynamic recruitment of ubiquitin to mutant huntingtin inclusion bodies. *Sci Rep* 8: 1405

104. Sun D, Wu R, Zheng J, Li P, Yu L (2018) Polyubiquitin chain-induced p62 phase separation drives autophagic cargo segregation. *Cell Res* 28: 405−415

105. Geurink PP, van Tol BD, van Dalen D, Brundel PJ, Mevissen TE, Pruneda JN, Elliott PR, van Tilburg GB, Komander D, Ovaa H (2016) Development of diubiquitin-Based FRET probes to quantify ubiquitin linkage specificity of deubiquitinating enzymes. *ChemBioChem* 17: 816−820

106. Ye Y, Blaser G, Horrocks MH, Ruedas-Rama MJ, Ibrahim S, Zhukov AA, Orte A, Klenerman D, Jackson SE, Komander D (2012) Ubiquitin chain conformation regulates recognition and activity of interacting proteins. *Nature* 492: 266−270

107. Bowman J, Rodgers MA, Shi M, Amatya R, Hostager B, Iwai K, Gao SJ, Jung JU (2015) Posttranslational modification of HOIP blocks toll-like receptor 4-mediated linear-ubiquitin-chain formation. *MBio* 6: e01777−01715

108. Sauer M, Heilemann M (2017) Single-molecule localization microscopy in eukaryotes. *Chem Rev* 117: 7478−7509

109. Sahl SJ, Hell SW, Jakobs S (2017) Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol* 18: 685−701

110. Betzig E (2015) Single molecules, cells, and super-resolution optics (Nobel Lecture). *Angew Chem Int Ed Engl* 54: 8034−8053

111. Gustafsson MG (1999) Extended resolution fluorescence microscopy. *Curr Opin Struct Biol* 9: 627−634

112. Heilemann M, van de Linde S, Schuttpelz M, Kasper R, Seefeldt B, Mukherjee A, Tinnefeld P, Sauer M (2008) Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. *Angew Chem Int Ed Engl* 47: 6172−6176

113. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF (2006) Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313: 1642−1645

114. Hell SW, Wichmann J (1994) Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt Lett* 19: 780−782

115. Chen BC, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW, Janetopoulos C, Wu XS, Hammer JA III, Liu Z *et al* (2014) Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* 346: 1257998

116. Furstenberg A, Heilemann M (2013) Single-molecule localization microscopy-near-molecular spatial resolution in light microscopy with photoswitchable fluorophores. *Phys Chem Chem Phys* 15: 14919−14930

117. Schnitzbauer J, Strauss MT, Schlichthaerle T, Schueder F, Jungmann R (2017) Super-resolution microscopy with DNA-PAINT. *Nat Protoc* 12: 1198−1228

118. Deussner-Helfmann NS, Auer A, Strauss MT, Malkusch S, Dietz MS, Barth HD, Jungmann R, Heilemann M (2018) Correlative single-molecule FRET and DNA-PAINT imaging. *Nano Lett* 18: 4626−4630

119. Huang B, Wang W, Bates M, Zhuang X (2008) Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science* 319: 810−813

120. Hands KJ, Cuchet-Lourenco D, Everett RD, Hay RT (2014) PML isoforms in response to arsenic: high-resolution analysis of PML body structure and degradation. *J Cell Sci* 127: 365−375

121. Hausmann M, Ilic N, Pilarczyk G, Lee JH, Logeswaran A, Borroni AP, Krufczik M, Theda F, Waltrich N, Bestvater F *et al* (2017) Challenges for super-resolution localization microscopy and biomolecular fluorescent nano-probing in cancer research. *Int J Mol Sci* 18: E2066

122. Yonezawa T, Takahashi H, Shikata S, Liu X, Tamura M, Asada S, Fukushima T, Fukuyama T, Tanaka Y, Sawasaki T *et al* (2017) The ubiquitin ligase STUB1 regulates stability and activity of RUNX1 and RUNX1-RUNX1T1. *J Biol Chem* 292: 12528−12541

123. Courtheoux T, Enchev RI, Lampert F, Gerez J, Beck J, Picotti P, Sumara I, Peter M (2016) Cortical dynamics during cell motility are regulated by CRL3(KLHL21) E3 ubiquitin ligase. *Nat Commun* 7: 12810

124. Gu ZC, Wu E, Sailer C, Jando J, Styles E, Eisenkolb I, Kuschel M, Bitschar K, Wang X, Huang L *et al* (2017) Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast. *Mol Biol Cell* 28: 2479−2491

125. Keller KE, Yang YF, Sun YY, Sykes R, Acott TS, Wirtz MK (2013) Ankyrin repeat and suppressor of cytokine signaling box containing protein-10 is associated with ubiquitin-mediated degradation pathways in trabecular meshwork cells. *Mol Vis* 19: 1639−1655

126. LaRock DL, Chaudhary A, Miller SI (2015) Salmonellae interactions with host processes. *Nat Rev Microbiol* 13: 191−205

127. Randow F, Youle RJ (2014) Self and nonself: how autophagy targets mitochondria and bacteria. *Cell Host Microbe* 15: 403−411

128. Perrin AJ, Jiang X, Birmingham CL, So NS, Brumell JH (2004) Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. *Curr Biol* 14: 806−811

129. Herhaus L, Dikic I (2017) Regulation of Salmonella-host cell interactions via the ubiquitin system. *Int J Med Microbiol* 308: 176−184

130. Cemma M, Kim PK, Brumell JH (2011) The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target Salmonella to the autophagy pathway. *Autophagy* 7: 341−345

131. Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, Brady NR, Richter B, Korac J, Waidmann O, Choudhary C *et al* (2011) Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. *Science* 333: 228−233

132. Thurston TL, Ryzhakov G, Bloor S, von Muhlinen N, Randow F (2009) The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol* 10: 1215−1221

133. Lork M, Delvaeye M, Goncalves A, Van Hamme E, Beyaert R (2016) Monitoring ubiquitin-coated bacteria via confocal microscopy. *Methods Mol Biol* 1449: 243−250

134. van Wijk SJ, Fiskin E, Dikic I (2013) Selective monitoring of ubiquitin signals with genetically encoded ubiquitin chain-specific sensors. *Nat Protoc* 8: 1449−1458

135. Manzanillo PS, Ayres JS, Watson RO, Collins AC, Souza G, Rae CS, Schneider DS, Nakamura K, Shiloh MU, Cox JS (2013) The ubiquitin ligase parkin mediates resistance to intracellular pathogens. *Nature* 501: 512−516

136. Huett A, Heath RJ, Begun J, Sassi SO, Baxt LA, Vyas JM, Goldberg MB, Xavier RJ (2012) The LRR and RING domain protein LRSAM1 is an E3 ligase crucial for ubiquitin-dependent autophagy of intracellular salmonella typhimurium. *Cell Host Microbe* 12: 778−790

137. Polajnar M, Dietz MS, Heilemann M, Behrends C (2017) Expanding the host cell ubiquitylation machinery targeting cytosolic Salmonella. *EMBO Rep* 18: 1572−1585

138. van Wijk SJL, Fricke F, Herhaus L, Gupta J, Hotte K, Pampaloni F, Grumati P, Kaulich M, Sou YS, Komatsu M *et al* (2017) Linear

ubiquitination of cytosolic salmonella typhimurium activates NF-kappaB and restricts bacterial proliferation. *Nat Microbiol* 2: 17066

139. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P, Zaffagnini G, Wild P, Martens S, Wagner SA *et al* (2016) Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci USA* 113: 4039 – 4044

140. Clough B, Wright JD, Pereira PM, Hirst EM, Johnston AC, Henriques R, Frickel EM (2016) K63-linked ubiquitination targets toxoplasma gondii for endo-lysosomal destruction in IFNgamma-stimulated human cells. *PLoS Pathog* 12: e1006027

141. Zhou Y, Dong N, Hu L, Shao F (2013) The Shigella type three secretion system effector OspG directly and specifically binds to host ubiquitin for activation. *PLoS One* 8: e57558

142. Fujita N, Morita E, Itoh T, Tanaka A, Nakaoka M, Osada Y, Umemoto T, Saitoh T, Nakatogawa H, Kobayashi S *et al* (2013) Recruitment of the autophagic machinery to endosomes during infection is mediated by ubiquitin. *J Cell Biol* 203: 115 – 128

143. Rahighi S, Ikeda F, Kawasaki M, Akutsu M, Suzuki N, Kato R, Kensche T, Uejima T, Bloor S, Komander D *et al* (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* 136: 1098 – 1109

144. Emmerich CH, Schmukle AC, Walczak H (2011) The emerging role of linear ubiquitination in cell signaling. *Sci Signal* 4: re5

145. Fiil BK, Gyrd-Hansen M (2014) Met1-linked ubiquitination in immune signalling. *FEBS J* 281: 4337 – 4350

146. Hacker H, Karin M (2006) Regulation and function of IKK and IKK-related kinases. *Sci STKE* 2006: re13

147. Fricke F, Beaudouin J, Eils R, Heilemann M (2015) One, two or three? Probing the stoichiometry of membrane proteins by single-molecule localization microscopy. *Sci Rep* 5: 14072

148. Hummer G, Fricke F, Heilemann M (2016) Model-independent counting of molecules in single-molecule localization microscopy. *Mol Biol Cell* 27: 3637 – 3644

149. Karathanasis C, Fricke F, Hummer G, Heilemann M (2017) Molecule counts in localization microscopy with organic fluorophores. *ChemPhysChem* 18: 942 – 948

150. Elson EL (2011) Fluorescence correlation spectroscopy: past, present, future. *Biophys J* 101: 2855 – 2870

151. Spahn C, Grimm JB, Lavis LD, Lampe M, Heilemann M (2018) Whole-cell, 3D and multi-color STED imaging with exchangeable fluorophores. *Nano Lett* 19: 500 – 505

152. Spahn CK, Glaesmann M, Grimm JB, Ayala AX, Lavis LD, Heilemann M (2018) A toolbox for multiplexed super-resolution imaging of the *E. coli* nucleoid and membrane using novel PAINT labels. *Sci Rep* 8: 14768

153. Heilemann M (2012) Elucidating cellular structures – superresolution microscopy. In *Comprehensive Biophysics*, Egelman E (ed). Amsterdam: Elsevier; ISBN 9780123749208.

154. Sauer M, Heilemann M (2017) Localization-based super-resolution microscopy. In *Fluorescence Microscopy*, Kubitscheck U (ed), pp 267 – 290. Weinheim: Wiley-Blackwell