MINIREVIEW

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Nanoscopy for endosomal escape quantification

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The successful cytosolic delivery of nanoparticles is hampered by their endosomal entrapment and degradation. To push forward the smart development of nanoparticles we must reliably detect and quantify their endosomal escape process. However, the current methods employed are not quantitative enough at the nanoscale to achieve this. Nanoscopy is a rapidly evolving field that has developed a diverse set of powerful techniques in the last two decades, opening the door to explore nanomedicine with an unprecedented resolution and specificity. The understanding of key steps in the drug delivery process – such as endosomal escape – would benefit greatly from the implementation of the most recent advances in microscopy. In this review, we provide the latest insights into endosomal escape of nanoparticles obtained by nanoscopy, and we discuss the features that would allow these techniques to make a great impact in the field.

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1. Introduction

Using nanoparticles (NPs) to deliver drugs to cells (nanomedicine) was foreseen to be a true game-changer of the 21st century in improving the prevention, diagnosis and therapy of various diseases.[1–6] The potential of these nanosized carriers in pharmaceutical applications has been envisioned since the 1970's to improve the delivery of therapeutic and imaging agents to specific target sites.[7–11] The remarkable interest in NPs is attributed to the plethora of physical and biological advantages they offer in comparison to conventional medicines, such as improved efficacy and safety, enhanced solubility and pharmacokinetic profiles, and increased target selectivity.[12–15]

Although various NP formulations have been marketed,[16,17] achieving efficient intracellular delivery still remains a significant challenge.[18–22] One of the main culprits is that the majority of NPs – once taken up *via* endocytosis – are unavoidably distributed in endocytic vesicles. These acidic organelles can degrade the carrier-drug ensemble, reducing its bioavailability

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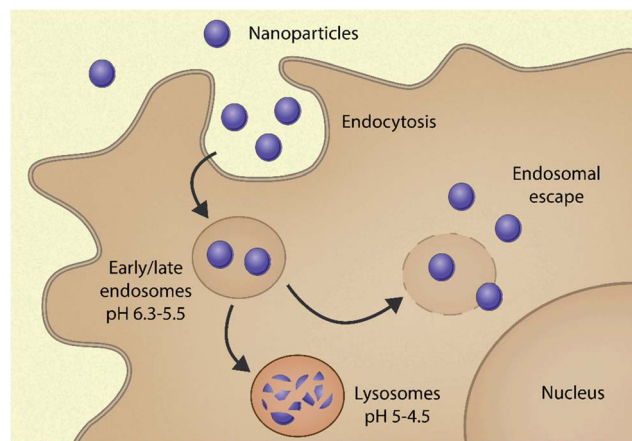
Fig. 1 Scheme of the route followed by nanoparticles inside the cell. They are first internalized by endocytosis into early endosomes, where they are trafficked through the endolysosomal pathway and ultimately degraded in the lysosomes. Nanoparticles escaped from endosomes to avoid degradation and deliver their cargo into the cytoplasm.

in the intracellular environment.[23,24] Within these vesicles, pH gradually drops from neutral to acidic because of membrane-incorporated vacuolar-type ATPases. The cargo is first brought into the early endosome (pH ~ 5.5); then finally the late endosome fuses with the lysosome and the cargo is degraded by hydrolytic enzymes present in the acidic milieu (pH 5–4.5). The recycling endosome may direct some cargo back to the cell surface, whilst the majority remain entrapped in the endolysosomal pathway, where they are degraded[20,25] (Fig. 1). Endosomal entrapment thus represents one of the main bottlenecks in using NP systems for gene therapy[20,26–28] and proteins or small molecular drugs for the treatment of a variety of diseases.[20,29–31]

Mechanisms through which NPs – and more importantly the therapeutic cargo – can escape these degrading vesicles have

become the subject of intense research over the past few decades.[19,20,24,32] Inspired by the innate ability of bacterial toxins and viruses to escape endosomal vesicles, various hypothetical endosomal escape mechanisms have been proposed and reviewed in the literature, such as the "proton sponge" effect, membrane fusion, pore formation, membrane disruption, and vesicle budding and collapse.[24,27,32–36] Numerous strategies to enhance the escape of NPs have also been suggested, including endosomal buffering agents, membrane fusogenic peptides, lysosomotropic chemical agents,[20,23,37–44] morphological-dependent changes[45] or external stimuli such as photochemical internalization (PCI).[46,47]

It is crucial to note that the endosomal escape hypotheses suffer from many inconsistencies. For example, the "proton sponge" hypothesis – based on the buffering capacity of polycations, that are suggested to cause an increase in lysosomal pH – has been heavily disputed in the literature.[48,49] As a result, the mechanism of the action of these formulation strategies is generally unknown. This is limiting the development of NPs with efficient endosomal escape, and it is further worsened by the absence of effective methods to detect – and more crucially to quantify – this process. Consequently, it is challenging to determine which strategies are efficient in improving the escape ability of NPs, hindering the development of successful formulations. Additionally, the lack of standardized methods leads to poor comparisons between different endosomal escape studies, leading to contradicting and inconclusive results.[48–53]

Standard methods used to assess endosomal escape commonly employ fluorescence microscopy, flow cytometry or mass spectrometry. However, fluorescence microscopy cannot be used alone to quantify the total number of particles inside cells, as this requires cumbersome calibration of the fluorescence signal, and lacks the resolution to quantify individual NPs below 250 nm. Flow cytometry measures relative fluorescence intensity rather than individual NPs, and mass spectrometry leads to the loss of spatial information.[54] Readers are directed to

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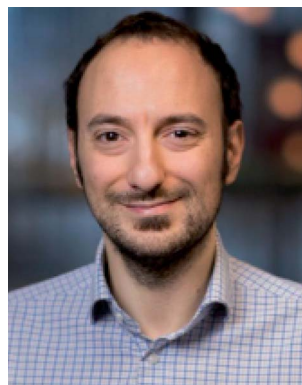
Dr Albertazzi obtained his PhD in Biophysics (2011) from Scuola Normale Superiore (Pisa, Italy). He then joined the Eindhoven University of Technology (TU/e, The Netherlands) as a postdoctoral researcher. In 2013, he was awarded a fellowship at the Institute for Complex Molecular Systems (ICMS) at TU/e, and in 2014 he became a NWO/VENI fellow. In 2015, he moved to IBEC to start the 'Nanoscopy for Nanomedicine' group. In 2018 he was appointed Associate Professor at the TU/e department of Biomedical Engineering. He is aiming to achieve a molecular understanding of synthetic materials in the biological environment, using optical microscopy and nanoscopy.

Table 1 Comparison of the selected characteristics of the nanoscopy techniques and confocal microscopy discussed, where bold indicates the best and italics the worst in each category

Technique	Resolution XY	Resolution Z	Live-cell imaging	Multi-colour	Temporal resolution	Quantification	Overall simplicity of technique	References
Confocal microscopy	~200 nm	~400 nm	Yes	Yes (3 colours)	ms–s	Worst	**Simple**	30,54,56,66 and 144–149
EM	**1 nm**	NA	No	No	**ms**	Good	Complex	18,30,58,60–62,64,66–74,76–78 and 150–153
SMLM	~20 nm	~80 nm	No[a]	Yes (2–124 colours)	min	Good	Complex	81–85
STED	~50 nm	~150 nm	Yes	Yes (3 colours)	s	Good	Simple	98–101
SIM	~100 nm	~300 nm	Yes	Yes (3 colours)	ms–s	Bad	Simple	107 and 112–115
CLEM	~1 nm (EM)	Dependent on LM technique	No	Dependent on LM technique	Dependent on LM technique	**Best**	Very complex	68,72 and 141–143

[a] SMLM does not allow live cell imaging in most cases, but there are few examples.[87,89]

other available reviews for information on these techniques and how they may compare with nanoscopy methods.[24,35,55,56] Studying endosomal escape brings alongside certain challenges; it is a fast process, rare and occurs in the nanoscale. All these techniques have limited spatial resolution, are often poorly quantitative and fail to provide information on endosomal escape at the nanoscale and quantitative level, or with high molecular specificity within the cellular biological environment. Therefore, new, and improved techniques are necessary for the quantification of NP–cell interactions to allow comparison and integration of data and push forward the smart development of NPs.

Here we highlight the most prominent nanoscopy techniques and discuss the features that overcome the limitations of standard methods. We briefly emphasize on how they can be used for quantification of endosomal escape, and we provide a short perspective on how these techniques can help us gain more insight into the process of NP endosomal escape, leading to the development of more effective formulations.

2. Discussion

As previously highlighted, endosomal escape is a process that is fast and rare and occurs at the nanoscale. Here we briefly discuss the pros and cons of various nanoscopy techniques that can be used to quantitatively study this process. For a summary of the techniques discussed see Table 1, and for extra information on how quantification can be achieved using these techniques, see Table 2. We put emphasis on the power of electron microscopy (EM), super-resolution microscopy (SRM) and correlative imaging to answer sought-after questions regarding NP endosomal escape, and ultimately on improving the development of NPs with efficient therapeutic cytosolic delivery.

2.1. Electron microscopy and cryo-electron microscopy

With a near atomic resolution,[57] EM is an irreplaceable tool in studying the physio-chemical properties of NPs and quantifying their voyage through the endo-lysosomal pathway.[30,37,58–74] EM can even detect a low number (few hundreds) of single nanoparticles escaping endosomal structures, and since it is a label-free method, it will localise and quantify NPs generally untraceable by standard light microscopy methods. TEM was demonstrated to quantify approximately 150-times more NP/cell compared to NP events/cell using a confocal laser scanning microscope.[75] EM allows direct visualisation and quantification of NPs and endosomal compartments and can distinguish between intracellular/extracellular/intramembranous NPs, both in 2D and 3D (Table 2).

As a pioneering example, Gilleron et al.[37] developed one of the most promising semi-automatic approaches using TEM, quantifying the amount of siRNA-conjugated colloidal gold NPs escaping from various endo-lysosomal compartments (Fig. 2A, top left). The authors developed a gold detection software that automatically detects and quantifies the total number of gold NPs in each image, based on the threshold intensity of gold.

Table 2 Overview of different types of quantification methods and how these can be achieved using nanoscopy and confocal microscopy, including information on throughput and disadvantages of the methods

Technique	Quantification	Quantification process	Throughput	Disadvantages
[a]Confocal	Co-localization	NP endosomal vesicles are tagged with different fluorophores and average fluorescence intensity is calculated (e.g. using ImageJ – Color2/JACoP). Co-localization calculated using Mander's/Pearson's correlation coefficients[56,148]	Fairly good throughput – (i.e. a few cells, tens of lysosomes and tens of NP clusters)	-Limited resolution -Cannot resolve individual NPs -Localization precision is affected by resolution
	Particle tracking	The total number of particles and endosome co-localized NPs is tracked and counted with particle tracking software. As NPs cannot be individually detected, particle events are calculated instead, whereby a single NP event likely corresponds to one vesicle containing NPs[75,154]		-Invisible particles (i.e. due to bleaching of fluorophores or de-coupling of fluorescent dyes) -Number of particles is underestimated -Choice of fluorophore can influence results (e.g. pH sensitive dyes can have reduced signals in acidic vesicles) -Fluorescence must be quantified in relation to a control to account for fluorophore instability
EM	Direct visualization and quantification	The ratio between NPs found in the cytosol and endosomes is calculated[148,155,156] Can distinguish between intracellular/extracellular/intramembranous nanoparticles	Low throughput – (i.e. one cell, tens of NPs and a few endosomes per field of view)	-Complicated sample preparation -Generally, samples are fixed and sectioned (i.e. no living cells)
	Serial sectioning or electron tomography	Imaging in 3D of sequential sample sections. Location, size, and the number of vesicles as well as NPs can be calculated in whole 3D cells[74,156]		-Difficulty in distinguishing different intracellular vesicles
	Stereological image analysis	Using the relative particle distribution within cells (RDI). Tests if NPs are localizing randomly or specifically within cellular compartments. The particle density of each compartment is calculated by relating the number of particle events in the specific compartment to the fractional volume of the compartment[157]		-Particles must be smaller than the section thickness (~150 nm)
		Correlating the total number of intracellular particles of a sample with the total cell number of that sample. Using the fractionator principle[54,158]		-Quantification from 3D reconstructions is difficult
		The density of intracellular particles is multiplied by the average cell volume to calculate the average number of NPs per cell[75]		-Restricted to samples with adequate atomic contrast

Table 2 (*Contd.*)

Technique	Quantification	Quantification process	Throughput	Disadvantages
SMLM	Spatial analysis and clustering	Single-molecule localization microscopy techniques produce point cloud data as a result of multiple localizations in time. These data can be analyzed to identify objects, and determine densities or spatial correlations[93]	Intermediate – low throughput. The field of view may vary from one to few cells. The imaging time would greatly depend on the specific technique used (seconds to minutes)	-In some cases, there are undesired non-specific interactions or background noise
	Molecule counting	Single-molecule localization microscopy techniques are based on the identification of individual molecules. Therefore, it is possible to quantify the exact number of molecules on a specific area.[159,160] For example, the ligands or proteins on the surface of a nanoparticle[161,162]		-High amount of data that can make the quantification process slow
	Stability of NPs and vesicles	The increased resolution and precise molecule counting of super-resolution microscopy allow the determination of the stability of small objects such as nanoparticles and vesicles. It is possible to establish their shape[96] and observe the degradation in time[99]		
STED/SIM	Size and shape of NPs	The improved resolution of these techniques allows the measurements of the size and shape of smaller objects compared to confocal microscopy[109,113,114]	Good throughput – imaging times in the millisecond-second range	-No single-molecule quantification
	Co-localization	Standard colocalization coefficient calculations are also applied for these techniques, although better resolution yields more precise results[109]		
CLEM	Combination of FM and EM techniques	Generally, fluorescence microscopy is carried out prior to EM. Images can be manually aligned using plugins such as eC-CLEM. Quantification can be achieved either *via* EM or FM, or both.[74,163] Detection of 'invisible particles' in light microscopy is possible with CLEM, as well as compartment-specific quantification	Low throughput – still limited by EM	-Complex and time-consuming sample preparation -NPs must be detectable using both light and electron microscopes -Alignment mismatch can affect correlation

[a] Quantification is not absolute (not at a single particle level) due to the resolution of the microscope.

Using this, they quantified the ratio of siRNA-gold within the endosomes and in the cytosol to calculate endosomal escape and found that only <2% of siRNA-gold escaped the endosomes in HeLa cells. Furthermore, using distinct mathematical models in combination with a pharmacological blockade of endosomal progression, they observed that release occurs
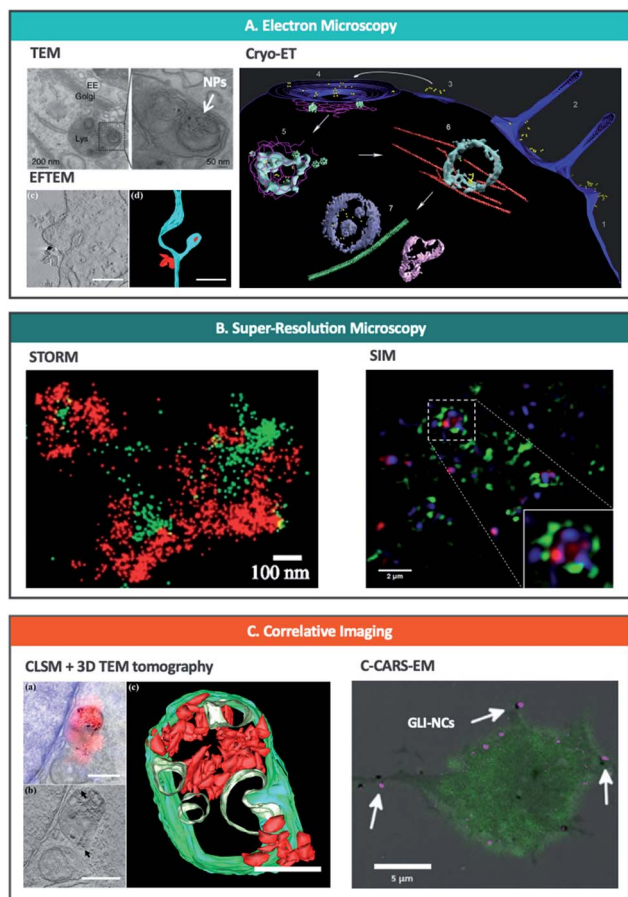
Fig. 2 Nanoscopy techniques used to study and/or quantify endo-lysosomal trafficking of nanoparticles. (A) Electron microscopy techniques including TEM (upper left),[37] EFTEM (lower left)[74] and Cryo-ET (right)[69] can be used to track and quantify nanoparticles in intracellular vesicles. Reprinted (adapted) with permission from ref. 37 Copyright © 2013 Nature America, Inc., from ref. 74 © 2019 American Chemical Society and from ref. 69 Copyright © Azubel *et al.* eLife. (B) Super-resolution microscopy has been used to image nanoparticles bursting out of endosomes with STORM (left)[96] and SIM (right).[117] Reprinted (adapted) with permission from ref. 96 and 117 Copyright 2018 American Chemical Society. (C) Correlative imaging combines different microscopic techniques such as CLSM and 3D TEM tomography (left)[74] or C-CARS and EM (right)[70] and offers spatiotemporal localization of labelled NPs and biomolecules with high specificity and sensitivity at a highly subcellular level; quantification possible with CLSM + 3D TEM tomography. Reprinted (adapted) with permission from ref. 74 Copyright © 2019 American Chemical Society and from ref. 70 Copyright © 2018 The Authors. Biotechnology Journal Published by Wiley-VCH Verlag GmbH & Co. KGaA.

mainly from the early endosome. Additionally, developments in staining methods such as photoconversion of diaminobenzene (DAB) – that allows the conversion of a fluorescent dye into an electron-dense signal – in combination with immunoelectron microscopy demonstrate that EM can be used to examine the interactions of NPs with cellular organelles and to detect if they are intact or degraded after endo-lysosomal breakdown.[76,77]

One of the limitations of conventional EM is that the image acquired corresponds to a distorted, dehydrated form of the natural specimen, due to the need for drying, staining or plastic

embedding the sample. Using cryo-EM, the specimen exists in a near-native frozen-hydrated state, maintaining the structures of interest as they would be in solution.[78,79] However, to date, the only paper exploiting cryo-EM to study the trafficking of NPs within the endosomal pathway (albeit indirectly) is by Azubel *et al.*[69] who employed cryo-electron tomography (cryo-ET) to study the endosomal trafficking of fibroblast growth factor 21 tagged to gold NPs (AuNP-FGF21) (Fig. 2A, right). By using 3D tomographic reconstruction, they were able to unequivocally identify gold NPs inside/outside various cellular structures including endosomes. Although the authors did not focus on quantifying the gold NPs, this technique has great potential to quantify the endosomal escape of various inorganic NPs, as well as that of different proteinaceous ligands/protein-based cargo at a single-particle level and with great localisation precision.

Indisputably, EM is an irreplaceable asset in the tracking and quantification of NP endosomal escape. However, it can only be used on fixed or frozen samples and it is inappropriate for studying dynamic changes. Cellular samples must be cut into <200 nm thin sections and exposed to various staining and washing steps that can lead to the loss of NPs.[80] Also, at the expense of high resolution, only a small field of view (a few endosomes and a few tens of NPs) can be analysed at one time, making this a low-throughput technique. Lastly, EM has reduced molecular specificity, thus making it difficult to distinguish between different types of vesicles within the endosomal pathway.[60]

2.2. Super-resolution microscopy or optical nanoscopy

In the history of light microscopy, better lenses were used to improve resolution by focusing more light onto the sample, such as the pinhole in confocal microscopy.[81] However, Abbe's diffraction's law[82] determines that the ultimate resolution of any light microscope is limited to 200–350 nm due to light diffraction. The advent of SRM[83] allows overcoming this limitation combining the advantages of fluorescence microscopy with nanometric resolutions. The specific labelling of proteins, multicolour ability and live-cell imaging at subcellular resolutions transformed this method into a new powerful tool to study endosome escaping.

2.2.1 Single-molecule localization microscopy (STORM, PALM and PAINT).
Single-molecule localization microscopy (SMLM) is a group of fluorescence SRM techniques based on the localization of single molecules with resolutions down to tens of nanometres. It was in 2006 when SMLM was introduced bringing in the idea of stochastically having only a sparse subset of the fluorophores 'on' at a time and repeating the process until the whole sample is analysed.[84-88] By superimposing those sparse single-molecule images we can reconstruct the initial image at a higher resolution. The difference between the various techniques relies on how they cause the fluorophores to switch between 'on' and 'off' states. Stochastic optical reconstruction microscopy (STORM)[86,89] and photoactivated localization microscopy (PALM)[85,87] are based on the photoswitching and photoactivation of organic dyes and fluorescent proteins respectively, and meanwhile point accumulation for imaging in nanoscale topography (PAINT)[88,90-92] is based on the binding

and unbinding of free diffusing fluorescent labelled probes to the target molecule.

The main advantages of these techniques are that they have an excellent resolution (5–25 nm) to visualize NPs and intracellular vesicles, and they can offer a powerful quantitative tool with single-molecule precision,[93] *i.e.* molecular counting. Moreover, they offer multicolour imaging, bringing in the possibility of labelling multiple subcellular structures as well as delivery carriers at the same time. In particular PAINT, by multiple rounds of imaging with different target probes[91,94] or by kinetic fingerprinting the binding interaction, has recently achieved 124 colour super-resolution imaging.[95] The main disadvantage of SMLM techniques is that they also require long imaging times to reconstruct the final image (few minutes to an hour), making them generally not suitable for live cell imaging.

Recently, STORM has been applied to observe endosomal escape of siRNA polyplexes[96] (Fig. 2B). In this study, they imaged polyplexes carrying siRNA in early and late endosomes with 2-colour STORM to directly visualize the rupture of endosomes and the release of polyplexes. They first measured the size of polyplexes in biological environments from 2D STORM images. Then, they observed the shape of individual endosomes and polyplexes inside cells to establish how the endosomal escape process was occurring. Finally, they combined 2-colour STORM images to determine the level of colocalization of polyplexes and endosomes by counting individual polyplexes. In fact, STORM has also been used recently to study the trafficking and stability of NPs in cells.[97–99] This and other techniques have shown the capability to image in 3D at the nanometric-scale resolution subcellular structures, such as endosomes and lysosomes, opening the door to a deeper understanding of endosomal escape.[100]

2.2.2 Stimulated emission depletion (STED). Stimulated emission depletion (STED) is a SRM technique initially proposed by Stefan W. Hell in the 90s[101] and firstly applied on biological samples in 2000.[102] It works by shrinking the excitation laser beam using a second doughnut-shape laser. This second beam depletes fluorescence and as a result only fluorescence from the centre of the doughnut is collected. The main advantage of STED is that it offers diffraction unlimited resolution at imaging speeds similar to a confocal – time resolution of seconds – as well as 3D and tissue imaging, as recently demonstrated by the imaging of NP internalization in 3D[103] and the crossing of the blood–brain barrier in brain tissue samples.[104] However, to effectively deplete fluorophore emission with a circular shaped beam, it requires a high intensity laser that may cause photodamage to cells,[105] although live-cell imaging can be carried out to some extent.[106]

STED nanoscopy has been applied in internalization and trafficking of NPs.[107–109] Specifically, Li Shang and co-workers investigated the internalization of transferrin NPs and measured the size of NP-loaded early endosomes with STED in live cells to conclude that particles were clustered inside the vesicles.[109] STED has not been used to date to study endosomal escape of NPs; however, due to the multiple advantages of this technique, we can foresee the potential of STED to contribute to this field.

2.2.3 Structured illumination microscopy (SIM). Structured illumination microscopy (SIM) is a SRM technique based on the Moiré effect, in which the sample is illuminated with a known pattern in different orientations and the resulting images can be deconvoluted into a higher resolution image.[110] SIM can achieve a resolution half of Abbe's theoretical limit, around 100–150 nm, as well as a fast imaging speed – below 1 second – and low light exposure to the sample compared to other SRM methods. This makes it the ideal SRM method for live-cell imaging.

SIM has had a great impact in studying cell–NP interactions due to its fast imaging speed, live-cell capabilities, and low restrictions on fluorophore selection. It has been applied to investigate NP internalization,[111] and trafficking[112] – as well as shape[113] and degradation[114] inside cells – and subcellular dynamic processes at few milliseconds time resolution.[115,116] Focusing on endosomal escape, SIM has been recently used to image the rupture of endosomes and the delivery of siRNA into the cytoplasm in breast cancer cells[117] (Fig. 2B). Moreover, SIM has revealed that PEI polyplexes are found close to the internal side of the membrane of lysosomes/late endosomes, rather than at a central position in the vesicle.[118] These findings prove the potential of SIM to investigate endosome–NP interactions in live cells, where an intermediate resolution is sufficient.

2.3. Frontiers in fluorescence micro/nanoscopy

The field of microscopy is constantly evolving and releasing new tools to tackle the challenges at the micro and nanoscopic scales. Recent developments have proven to be powerful techniques to study NPs in the biological environment offering better resolution and live-cell imaging features. Specifically, AiryScan[119] and RESOLFT[120] came into play to reduce photobleaching in confocal and STED microscopy respectively, for improved live-cell imaging, dynamic studies and higher throughputs. Moreover, recently developed MINFLUX (minimum photon fluxes) has achieved an outstanding resolution of 1–3 nm with low laser exposure in 3 dimensions.[121,122]

2.4. Dynamic imaging

Endosomal trafficking and escape of NPs not only occur at the nanoscale, but are also a dynamic process. Some microscopy techniques can be combined with other tools to further investigate dynamic processes, such as fluorescence resonance energy transfer (FRET),[123–125] fluorescence correlation spectroscopy (FCS)[126,127] and single-particle tracking (SPT).[128–132] Interestingly, SPT has been extensively used to study the co-localization and quantification of NPs within endocytic vesicles.[128,133–139] For instance, Zahid *et al.*[133] used live-cell SPT in combination with multidimensional analysis to characterize the intracellular distributions of quantum dot (QD) properties and to quantify their endosomal escape. The knowledge provided by SPT data analysis – especially when combined with other techniques – can be used to understand the underlying biological mechanisms of what discriminates formulations that achieve endosomal escape from those that cannot.

2.5. Correlative imaging

Various papers report the endosomal escape of NPs using several independent microscopic techniques.[30,58,68,140,141] However, a correlative approach is more desirable, as it bridges the advantages of two distinct techniques by imaging the same region of interest and overlapping important information from the two methods. Despite a much greater image interpretation confidence – that would not be possible with either of the methods individually[142] – there is a very low number of publications in the area. This is probably related to the complex and cumbersome procedures required for sample handling and image aligning.[143]

Correlative light and electron microscopy (CLEM) are perhaps the most explored group of correlated techniques. This combination allows spatiotemporal localization of labelled biomolecules with high specificity and sensitivity (FM), and with (sub)-nanometer resolution and precise subcellular localization of NPs within the cell (EM). In practice, quantification precision can be greatly improved using CLEM, as 'invisible particles' (*i.e.* not labelled with a fluorescent dye/not electron dense enough) can be detected. Also, since it can be difficult to distinguish different endosomal compartments based just on the TEM morphology, correlation with fluorescently labelled compartments in light microscopy can also improve NP localization precision.

To date, the only CLEM approach used to quantitatively study endo-lysosomal tracking of NPs has been developed by Han *et al.*[74] (Fig. 2c, left). Using confocal laser scanning microscopy (CLSM) and 3D TEM tomography, they were able to demonstrate the localization of fNDs within endosomes, lysosomes and autophagosomes. Using the high-resolution TEM tomography results, they precisely quantified single fNDs found in clusters within the endosomal vesicles. However, quantification of single fNDs (not within clusters) was only possible at the single-particle level by using EFTEM (energy filtered TEM) as an additional method (Fig. 2A, bottom left). Furthermore, EFTEM permitted autonomous TEM screening of the whole sample, demonstrating the potential of this technique to precisely identify and quantify intracellular NPs.

Haruta *et al.*[144] used the local surface plasmon resonance (LSPR) of gold NPs as a tag for biological samples in CLEM. To alleviate the problem of the resolution mismatch of several orders of magnitude between the two techniques, EM has also been correlated with SRM. Fluorescent nanodiamonds (FNDs) have been studied at nanometer resolution using STED-TEM[145] and integrated light and scanning EM.[146] However, in these examples NPs have been used for correlative purposes rather than to quantify or answer specific questions regarding intracellular trafficking. SRM-EM in fact offers a powerful tool to quantify and track endosomal escape and research in this area would benefit greatly the nanomedicine community.

A more distinct approach was achieved by Saarinen *et al.*[70] who used correlative coherent anti-Stokes Raman scattering and TEM (C-CARS-EM) to image glibenclamide-nanocrystals (GLI-NCs) in macrophages (Fig. 2c, right). The combination of a label-free and chemically specific C-CARS technique with the excellent resolution and precision of TEM, allowed precise localization of GLI-NCs within endosomal vesicles. Although not achieved in this work, this technique also has the potential to be quantitative. For example, using 3D information from C-CARS together with precise localization of nanocrystals from EM, one can calculate the ratio between NPs found in the cytosol and in the endosomes (endosomal escape).

3. Conclusions and perspectives

Whilst significant progress has been made on developing a rich formulation databank of NPs for cytosolic delivery,[16,17] our understanding of the physiochemical and biological requisites for achieving endosomal escape has been hampered.[20,26–31] Our grasp of these mechanisms is hampered by the limitations of the standard techniques used to localise and quantify them. As discussed in this review, nanoscopy techniques – independently, or in correlation – hold the promise of answering some essential questions regarding endosomal escape. Some of these questions include how and which physiochemical properties of NPs influence endosomal escape? Which of the proposed endosomal escape mechanisms stand true and how can we improve the formulation of NPs to exploit them? Can we relate endosomal escape to the time and location at which it occurs intracellularly?

Here we have highlighted the relevance of nanoscopy and some of the most recent discoveries in endosomal escape possible only using these methods. With a plethora of advanced microscopic techniques available, it is essential that we weigh the pros and cons of each technique to best suit the scientific question proposed (Tables 1 and 2). For a process such as endosomal escape – that is fast and rare and occurs at the nanoscale – it may seem challenging to answer the various questions projected using individual methods. But as we have seen in this review, we are no longer restricted to a 'one method at a time' approach.[70,74,142,144–146] The benefits of correlative imaging – especially of SRM-EM – are of tremendous relevance in obtaining quantitative information on NP endosomal trafficking. Furthermore, as the amount of imaging data is increasing, automated quantification is becoming crucial in reducing manual analysis of images (and increasing throughput) and extracting more valuable data found in microscopic images, as well as making these techniques available to a broader research community.

Overall, these new developments in the field of imaging will lead to exciting times ahead for the study of endosomal escape. We prompt the nanomedicine community to adopt the newest techniques available to achieve a better understanding of NP trafficking as well as to facilitate the rational design of NPs with the ability to overcome endosomal barriers.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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