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Advanced optical imaging for the rational design of nanomedicines

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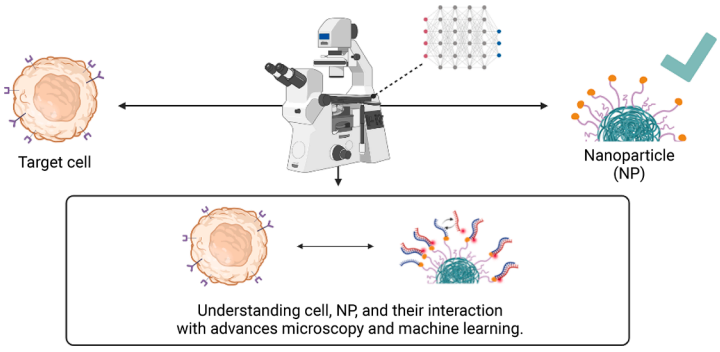
GRAPHICAL ABSTRACT



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ABSTRACT

Despite the enormous potential of nanomedicines to shape the future of medicine, their clinical translation remains suboptimal. Translational challenges are present in every step of the development pipeline, from a lack of understanding of patient heterogeneity to insufficient insights on nanoparticle properties and their impact on material-cell interactions. Here, we discuss how the adoption of advanced optical microscopy techniques, such as super-resolution optical microscopies, correlative techniques, and high-content modalities, could aid the rational design of nanocarriers, by characterizing the cell, the nanomaterial, and their interaction with unprecedented spatial and/or temporal detail. In this nanomedicine arena, we will discuss how the implementation of these techniques, with their versatility and specificity, can yield high volumes of multi-parametric data; and how machine learning can aid the rapid advances in microscopy: from image acquisition to data interpretation.

Abbreviations: MTT, 5-diphenyltetrazolium bromide 3-(4,5-dimethyethiazol-2-yl)-2; BrdU, 5-bromo-2′-deoxyuridine; AB, Antibody; ABs, Antibodies; AFM, Atomic Force Microscopy; CTC, omputerized Tomography; CLEM, Correlative Light and Electron Microscopy; DDS, Drug Delivery System; DDSs, Drug Delivery Systems; dSTORM, direct Stochastic Optical Reconstruction Microscopy; DLS, Dynamic Light Scattering; EPR, Enhanced Permeation and Retention; EGFR, Epidermal Growth Factor Receptor; HCI, High-Content Imaging; ML, Machine Learning; MRI, Magnetic Resonance Imaging; MERFISH, Multiplexed Error-Robust Fluorescent *in situ* Hybridization; NP, Nanoparticle; NPs, Nanoparticles; NIR, Near Infrared Radiation; PAINT, Points Accumulation for Imaging in Nanoscale Topography; PSF, Point Spread Function; PLGA-PEG, Polylactic polyglycolic acid-polyethylene glycol; PET, Positron Emission Tomography; PI, Propidium Iodide; RESI, Resolution Enhancement by Sequential Imaging; ROS, Reactive Oxygen Species; SUM-PAINT, Secondary label-based Unlimited multiplexed DNA PAINT; SMT, Single-Molecule Tracking; SPT, Single-Particle Tracking; SIM, Structured Illumination Microscopy; STED, Stimulated Emission Depletion; SMLM, Single Molecule Localization Microscopy; SR, Super-Resolution; SRM, Super Resolution Microscopy; TIRF, Total Internal Reflection Fluorescence; TEM, Transmission Electron Microscopy..

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1. Introduction

Drug delivery refers to the safe transport of a therapeutic agent to a target tissue, cell or organelle, to carry out a specific function [1]. Nanoparticle (NP)-based Drug Delivery Systems (DDSs) [2] have been developed for many applications in the biomedical field, including cancer treatment [3], immunotherapies [4] or disease prevention [5]. The use of these nano-vehicles offers many advantages over traditional therapy (or *free drug*): from safer and more efficient therapies [3], to novel therapeutic strategies, such as mRNA vaccines [6]. NP-based DDSs or nanomedicines provide a controlled release of therapeutic agents [1], which can be co-administered in the same platform, or combined with an imaging agent in a theragnostic device [7]. Moreover, nanocarriers allow the delivery of agents that cannot be administered on their own (such as poorly soluble drugs [8], nucleotide therapeutics [9], enzymes [10], etc.) to a specific target.

Nanoparticles tend to accumulate preferentially in organs with larger blood irrigation and bigger capillary fenestrations, such as liver and spleen, and certain solid tumors, with additional impaired lymphatic drainage. This pathophysiological phenomenon is coined as Enhanced Permeation and Retention (EPR) [11]. The ability to target can therefore be achieved intrinsically by the carrier's size (*passive targeting*), but also by decorating its surface with ligands that have affinity to a specific receptor expressed in the cell (*active targeting*) or by engineering the NP surface in a way that acquires a biomolecular corona with affinity to a certain target tissue (*endogenous targeting*) [12]. From antibodies to small molecules, a wide variety of ligands are available to drive NP fate [13,14]. In addition, smart nanomedicines can also be engineered to respond to local changes in the environment (e.g., pH, redox) or external stimuli (e.g., light, temperature, magnetic field); influencing cargo release and drug biodistribution [15]. These highly tunable systems are amenable for precision medicine [16], in which a specific cocktail of drugs, together with a suitable targeted vehicle, could be adjusted based on the patient's (or patient group) unique profile.

As it happens with novel drugs, the development of nanoparticles is a complex landscape, subjected to strict regulation [17]. In more than 30 years of nanomedicine research, roughly 30 candidates have reached the clinic (29 candidates in 2021, including the emergency approvals of the COVID vaccines [18]). Although the COVID-19 pandemic has undoubtedly stimulated the development of novel NP-based platforms, the nanoparticle development funnel remains pronounced and the clinical translation rate suboptimal [18,19]. Failure to enhance treatment efficacy and unexpected toxicities are the main reasons for NP withdrawal from clinical trials [20,21]. Lack of improved efficacy is often attributed to the limited or suboptimal nanoparticle accumulation in targeted tissue (on average $< 0.1\%$ of the administered nanomaterial in solid tumors) [22]. On one hand, the EPR effect is highly heterogenous between solid tumors and among patients [23]. On the other hand, the complexity of the human EPR is not well represented in pre-clinical models [11,23]. In essence, translational challenges of different nature are present in every step of the development pipeline from lab bench to bedside [24,25]. These barriers include the suboptimal ability of conventional pre-clinical models to replicate key aspects of the human disease [26], lack of understanding of patient heterogeneity and unproper patient stratification [27,28], insufficient fundamental or mechanistical understanding of NP interactions with living systems [25] or scale-up manufacturing of nanocarriers [29], among others. How nanoparticles interact with biological systems and overcome the biological barriers standing between them and their target is greatly influenced by their physicochemical properties, route of administration and patient's particular traits [16,21,30]. It is not surprising that understanding the relationships between material properties and its biological performance has become central to the rational design of novel nanomedicines. Unravelling this information is, however, far from trivial.

Optical microscopy has been widely used to understand cells and materials [31,32]. However, classic optical microscopy is still restricted by some limitations in its resolution, throughput, and quantitation. The spatial resolution in conventional fluorescent microscopy (i.e., widefield and confocal) is limited by the optical components of the microscope and the wavelength of the light used. Here, there is a physical limit imposed by the diffraction of visible light, known as Abbe or Rayleigh criterion [31], which sets the limit between resolvable objects to approximately 250 nm. Interrogating single nanoparticles and their physicochemical properties with nanometric detail was out of the scope of conventional diffraction-limited fluorescent microscopy. Visualizing cellular ultrastructure or nanoparticle size and morphology was only possible with electron microscopy, until the development of Super-Resolution (SR) optical microscopy. These advanced optical modalities can go beyond the diffraction limit of light to resolve cellular structures and materials with unprecedented detail [33-35]. The combination of automation, imaging and data analysis opens the way to high-throughput and high-content imaging, extending the possibilities to study large libraries of nanomedicines. Lastly, novel developments in image analysis and artificial intelligence nicely complement these novel optical techniques, allowing a more quantitative and powerful readout. We believe these methods will bring a deeper understanding of NP's physicochemical properties, including particle heterogeneity, and their interactions with cells; supporting the rational design of nanomedicines.

In this review, we will discuss how advanced optical imaging techniques can aid the development of novel nanomedicines, focusing on the initial stages of the nanomedicine development: the discovery [36]. In a generalized (and simplified) framework for NP discovery (Fig. 1A), after the definition of a problem, i.e., a disease target cell type with specific biochemical features, a few NP candidates are proposed based on the knowledge of the diseased cells (e.g., presence of biomarkers). These NP candidates are formulated, characterized and screened *in vitro*. After this 'round', a new set of candidates can be formulated, repeating the process (trial and error) until finding a NP candidate (or candidates) that fulfills the application and can be further developed.

This traditional way of exploration still faces several key challenges. First, for highly tunable particles, such as lipid-based or polymeric NPs, the potential number of NP compositions and features are remarkably high [37,38]. Therefore, formulating, characterizing, and testing all possible combinations may not be feasible, neither cost- nor timewise. High-throughput methods aim to overcome this particular challenge. High-throughput formulation, characterization and testing can already be achieved in some cases by automated workflows involving liquid handling robots, microfluidic-based systems and, more recently, Machine Learning (ML) [39-41]. Second, the most standard methodologies for NP analysis, such as Dynamic Light Scattering (DLS), are bulk measurements that fail to unmask nanoparticle heterogeneity and resolve the carrier's structure. This is also the case for cell interrogation, in which cellular heterogeneities had only been revealed with the advent of single-cell analysis techniques [42-44]. Adoption of advanced optical techniques in different steps of the nanomedicine development pipeline can not only help interrogating the nanocarrier structure [45] or cell features [46,47] with single-molecule precision, but also capture the complexity of their interactions [34]. The implementation of high-throughput methodologies together with advanced optical techniques will accelerate the pace, volume and complexity of the data generated, surpassing human capabilities to handle, process and interpret data without the aid of ML. Therefore, we identified three primary areas where advanced optical imaging can contribute to the NP discovery process (sections 2.2, 2.3 and 2.4, Fig. 1B), which can be powered by ML (section 2.5, Fig. 1C):

In **section 2.2**, **'understanding the cell'**, we will discuss how a family of optical techniques, Super Resolution Microscopy (SRM), have unlocked new opportunities in understanding the cell. How SRM is able to spatially resolve cellular structures [46,48,49] and single entities like receptors or protein biomarkers, which can be tracked and quantified with high sensitivity [50,51]. A better understanding of the disease

target will guide the design of a better library of particles to be tested.

In **section 2.3, 'understanding the material'**, we will see how this same family of techniques (SRM), alone or in combination with other high-resolution methods (e.g., correlative imaging), can help us characterize NP's properties at a single-particle level and with nanometric detail. This includes ligand quantification, distribution, and functionality [52,53], but also other NP physicochemical properties like shape or hydrophobicity [54,55].

In **section 2.4, 'understanding the cell-material interaction'**, we will discuss a wider range of optical modalities, from widefield to SRM, that can be used to interrogate different aspects of safety [56,57] and efficacy of nanocarriers [58-60], providing a better understanding of the material-cell interactions.

In **section 2.5, 'machine learning in advanced microscopy'**, we

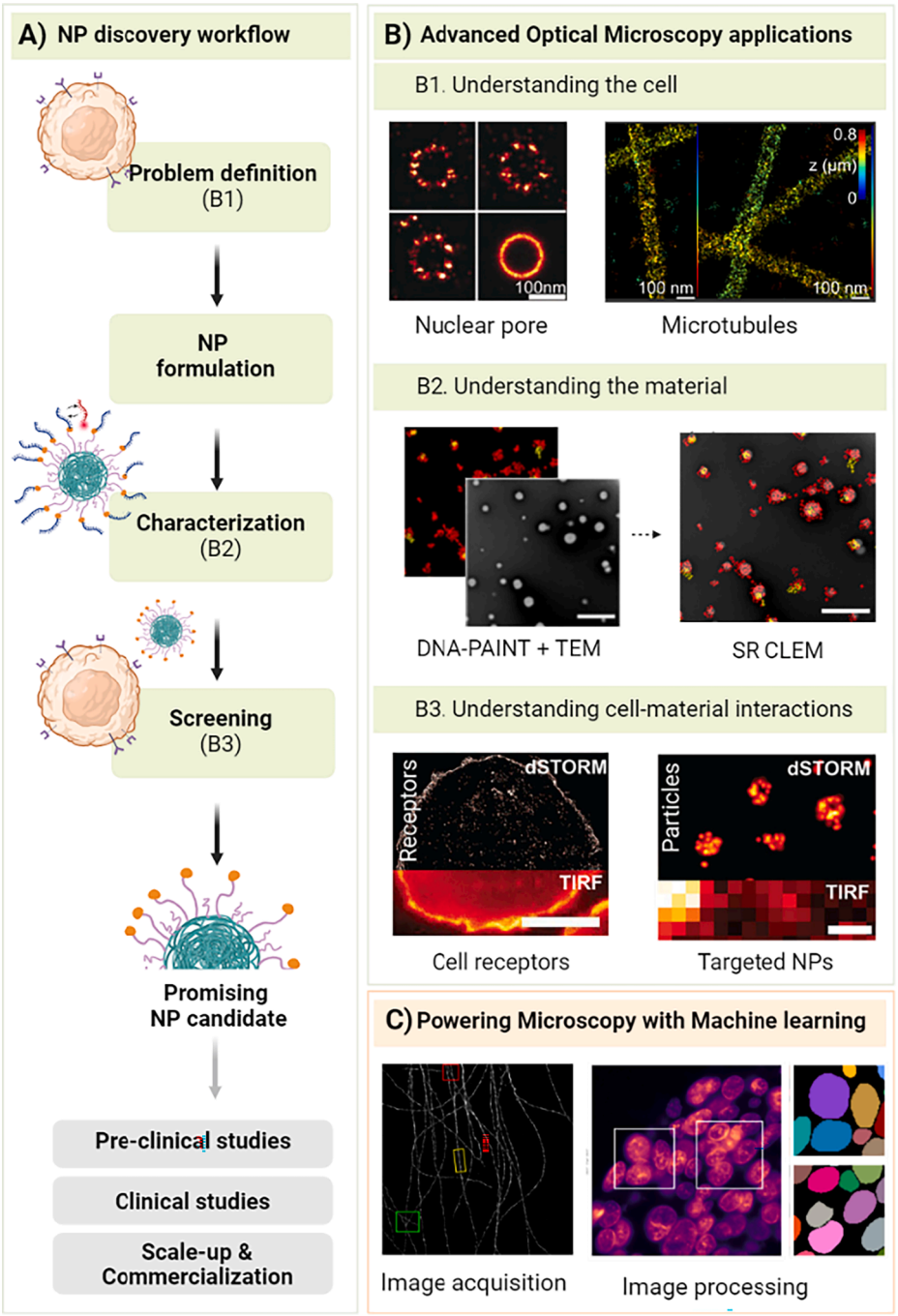

Fig. 1. Advanced optical imaging for the rational design of nanomedicines. A) Simplified nanoparticle (NP) discovery workflow to find a suitable or promising NP candidate for a given target diseased cell. B) How Advanced optical microscopies can be integrated in different steps of the NP development workflow to aid the rational design of future nanomedicines. Examples of optical imaging. B1) Understanding the cell, by resolving cellular structures (e.g., nuclear pore, microtubules) with up to single-molecule resolution. Reproduced from [46] and [48], respectively). B2) Understanding the material, by quantifying ligand number, distribution, accessibility. Adapted from [61]. B3) Understanding cell-material interactions, by investigating the correlation between cell receptor and number of ligands on targeted NPs. Adapted from [60]. C) Powering microscopy with Machine Learning (ML), by optimizing image acquisition (adapted from [62]) and image processing (e.g., object segmentation) (adapted from [63]). Abbreviations: Points Accumulation for Imaging in Nanoscale Topography (PAINT), Transmission Electron Microscopy (TEM), Super-Resolution (SR), Correlative Light and Electron Microscopy (CLEM), direct Stochastic Optical Reconstruction Microscopy (dSTORM), Total Internal Reflection Fluorescence (TIRF).

will touch upon some advances in ML approaches that can aid the acquisition and analysis of this imaging data. Overall, we believe that the adoption of these techniques can improve our understanding of nanomedicines and biological systems and, in return, generate useful information to drive the rational design of future nano-therapies.

2. Advanced optical microscopy in nanomedicine development

2.1. A brief introduction to Super resolution microscopy (SRM)

Together with well-stablished widefield and confocal imaging (conventional or diffraction-limited fluorescence microscopy), new methods have emerged in the last decade, extending the resolution with which cells can be probed [33-35]. Super Resolution Microscopy (SRM) is the name given to the family of fluorescent imaging techniques that go below Abbe's diffraction limit [33]. Rather than looking at whether a cell is potentially positive or not for a target of interest, SRM enables not just the identification and localization of sub-cellular, or even sub-organelle targets, but also their orientation and abundance with great sensitivity and specificity [47,64-77]. Breaking through this barrier lead to the Nobel prize in Chemistry in 2014 for Moerner, Hell and Betzig. The three main SRM families include Structured Illumination Microscopy (SIM) [78], Stimulated Emission Depletion (STED) [79] and Single Molecule Localization Microscopy (SMLM) [80]. They are classified based on type of illumination used and the strategy to build the super-resolved image: by varying the illumination pattern (SIM), by using an excitation beam in combination with a depletion donut-shaped beam (STED) or by exciting sub populations of fluorophores over many frames (SMLM) (Fig. 2). For a detailed explanation of these techniques, we refer our readers to the following reviews [33-35].

In this review, we want to highlight the potential applications of SRM to support nanomedicine development. We mostly focus on SMLM techniques, specifically on variants of direct Stochastic Optical Reconstruction Microscopy (dSTORM) [81] and Points Accumulation for Imaging in Nanoscale Topography (PAINT) [82]. Both dSTORM and PAINT are SMLM techniques that are usually accomplished in Total Internal Reflection Fluorescence (TIRF) microscopy. Although their implementation with other types of illumination is emerging [83]. In dSTORM, the image is reconstructed over time by detecting individual fluorescent dyes, which blink as a result of cycling between ON and OFF states randomly (stochastically) over many frames. Whereas in PAINT, individual fluorescent responses are a result of transient binding events between a probe and its target. The first and most utilized variant is DNA-PAINT [84], which uses a pair of complementary docking and imager DNA strands as probe. The docking strand binds to the target, while the complementary imager carrying the fluorophore hybridizes with the docking. This transient binding is enabled thanks to the low affinity binding of the probe. Recently, other low affinity probes, such as aptamers or peptides, have also been developed due to the advantages of small probe size and genetic encodability for living cell imaging [85]. It is clear that the choice of probe and fluorophore depends on the nature of the experiment (e.g., fixed vs. living cells) and the microscope modality. Fluorescent probes are selected based on their physicochemical and optical properties. These include size, fluorescence spectra, brightness, photostability, intermittent blinking behavior or capacity to label a specific target, among others. For each SRM modality, the specific requirements vary. While STED can benefit from dyes with high photostability due to the high power of the depletion beam, intermittent

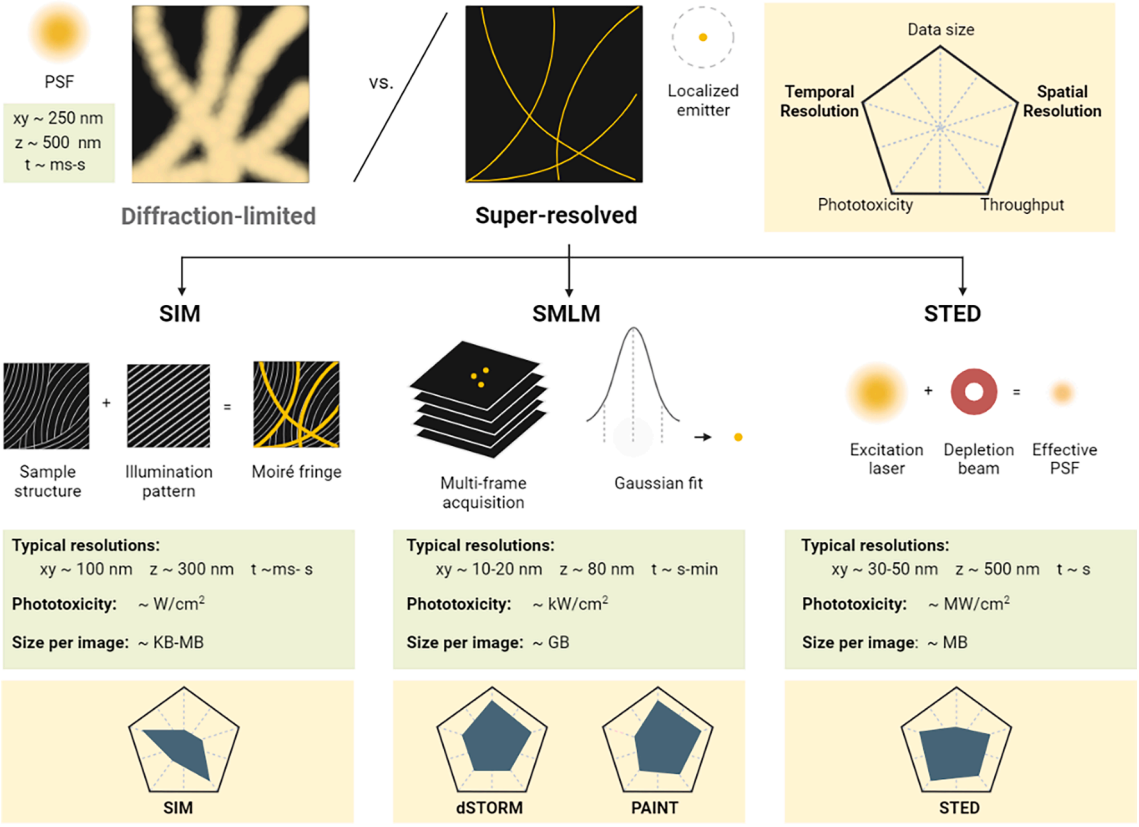

Fig. 2. Comparison between diffraction-limited fluorescence microscopy and main Super Resolution Microscopy (SRM) techniques. Schematic representations of working principles of the three main SRM families: Structured Illumination Microscopy (SIM), Single Molecule Localization Microscopy (SMLM) and Stimulated Emission Depletion (STED). Typical average resolutions (lateral, axial, temporal), phototoxicity and size per image. Schematic radar plots comparing the main techniques in terms of data size, temporal resolution, spatial resolution, phototoxicity and throughput. Abbreviations: Point Spread Function (PSF), direct Stochastic Optical Reconstruction Microscopy (dSTORM), Points Accumulation for Imaging in Nanoscale Topography (PAINT).

blinking is mandatory in SMLM and localization precision can be enhanced with brighter dyes. We refer our reader to review papers dedicated to fluorescent probes for different SRM modalities [85-89].

2.2. Understanding the cell

Imaging cells to characterize and quantify their inherent properties has become a cornerstone across multiple fields. This is also a crucial step of the NP discovery process, in which a rational design of nanocarriers can benefit from having prior knowledge about the specificities of the cells to be targeted. For instance, optical imaging can be used to determine the expression of biomarkers, receptors or drug targets [50,64,68]; as well as shed light on cellular structures, tissue organization and their changes upon biochemical perturbations [46-48,65,77]. Here, we discuss how these new methods can push the current boundaries of spatial and temporal resolution, as well as sensitivity and multiplexing of cell imaging.

Spatial resolution. The chemical specificity that characterizes fluorescence microscopy together with the ability of resolving objects with nanometric scale, makes SRM suitable to reconstruct a detailed and informative snapshot of the cell. Quantitative expression of drug targets [64], their precise sub-cellular location [90], or even their structure and organization [46,49] can now be revealed with SRM. Spatially resolved methods are primarily concerned, but not limited to fixed samples imaged with SRM. SMLM techniques, such as dSTORM [67] and PAINT [74], can resolve cellular structures in exquisite detail (<20 nm), but usually at a moment frozen in time (Fig. 3A1). SRM techniques provide a significant advantage over more traditional methods, especially concerning targets for precision nanomedicine: their single-molecule sensitivity. In this regard, Nerreter *et al.,* [64] demonstrated higher sensitivity of CD19 imaging using SRM in comparison to flow cytometry. Using SRM Nerreter *et al.,* were able to profile the expression of ultra-low levels of CD19 in melanoma cells and correlate it with the efficacy of the immunotherapy; highlighting the potential of SRM for patient selection. Apart from their characteristic sensitivity and specificity, SMLM techniques can image multiple targets on the same sample. Multiplexing, with regards to SRM, is the repeated application of a selection of fluorescent targets imaged in the same sample. This way it is possible to build up a near complete image of the cell with tens of nm spatial resolution using multiplexed dSTORM [67]; multiplexed PAINT (e.g. exchange-PAINT, universal PAINT) [66,92], or Multiplexed Error-Robust Fluorescent *in situ* Hybridization (MERFISH) [93]. While multiplexed dSTORM and PAINT enable spatial proteomics (targets are usually proteins), MERFISH allows spatial transcriptomics or RNA profiling [93]. Moreover, these techniques are not limited to 2D: lateral and axial resolutions of sub-20 nm and sub-80 nm have been reported with 3D multiplexed PAINT[83]. In 2023, Unterauer *et al.*, introduced a novel modality for exchange-PAINT that allows the mapping of virtually unlimited number of targets with a 15 nm resolution: Secondary label-based Unlimited multiplexed DNA PAINT (SUM-PAINT) (Fig. 3A2). SUM-PAINT was used for spatial proteomics, to map 30 protein targets in neurons [90]. From the perspective of personalized nanomedicine, these techniques can be exploited to characterize patient biopsies, across a library of potential biomarkers, revealing local organization and relative abundances. Diseases such as cancer can be assessed at an individual level, guiding medical responses towards more personalized treatments. Recent advances have pushed the spatial resolution down to Ångström levels [49]. Using a modified version of exchange-PAINT

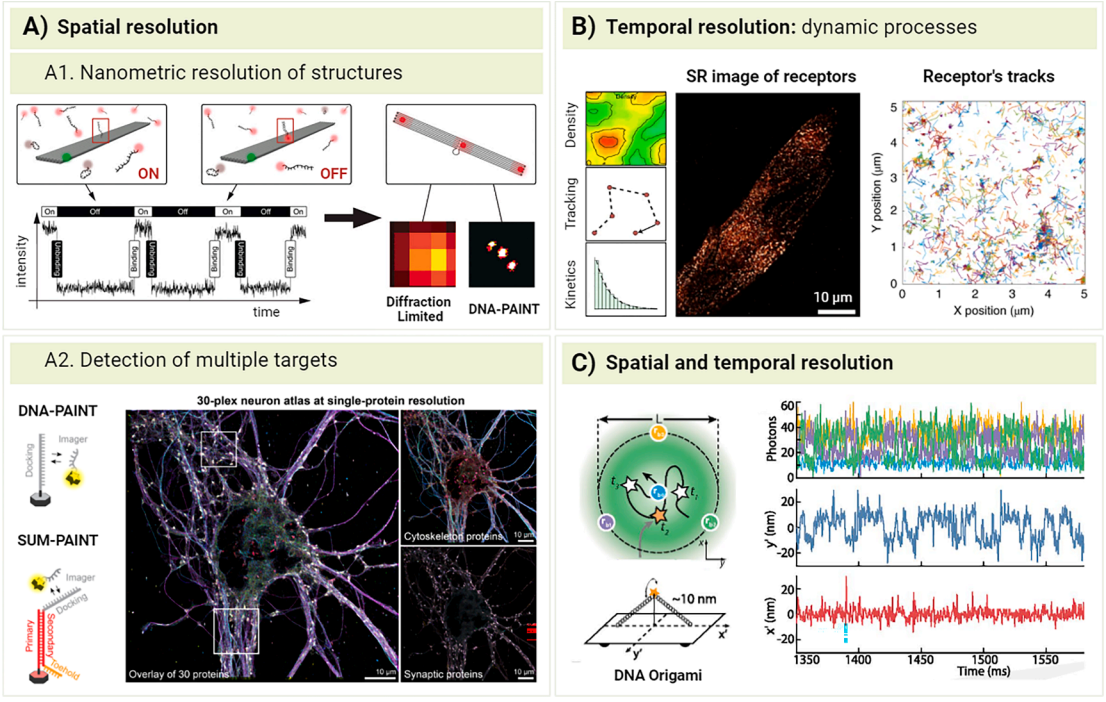

Fig. 3. Super resolution microscopy (SRM) to understand the cell. A) Spatial Resolution. A1) Schematic representation of DNA-PAINT working principle, typical time trace showing blinking behavior and comparison in resolution between a diffraction-limited spot and standard DNA-PAINT (<30 nm). Adapted from [84]. A2) Detection of multiple targets. Probe design of SUM-PAINT vs standard DNA-PAINT. Multiplexing of 30 neuron proteins (cytoskeleton and synaptic proteins) to build a 'neuron proteomic atlas'. Adapted from [90]. B) Temporal resolution. Glyco-PAINT, a temporally-resolved PAINT method using glycan probes to study glycan-receptor interactions in cells (i.e., receptor density, tracking and binding kinetics) with tens of ms of temporal resolution. Reconstructed Super-Resolution (SR) image of events on a cell (scalebar 10 µm). Receptor trajectories on cell surface. Adapted from [50]. C) Spatial and temporal resolution. MINFLUX, a SMLM-STED hybrid technique, allows the detection of an emitter inside the donut-shaped beam with minimum photon budget. MINFLUX was used to track the movement of a DNA origami construct, resolving fluorophore localization (x,y) within ~ 2 nm in the millisecond range. Adapted from [91]. Abbreviations: Point Accumulation for Imaging in Nanoscale Topography (PAINT), Secondary label-based Unlimited multiplexed DNA PAINT (SUM-PAINT), Single Molecule Localization Microscopy (SMLM), Stimulation Emission Depletion (STED).

based on DNA barcoding (Resolution Enhancement by Sequential Imaging (RESI)), single-protein resolution was achieved in intact cells. RESI was then used to study the arrangement of CD20, a therapeutic target in autoimmune diseases and blood cancers.

Temporal Resolution. Spatially resolved techniques, like dSTORM and PAINT in 'fixed mode', provide the highest resolutions from the SRM toolkit. However, a single image can take several minutes, which limits their application to study cellular processes. Temporally resolved methods enable the investigation of the dynamics of a cellular system and its evolution, achieving acquisition speeds from s to ms. Here, spatial resolution is often sacrificed to facilitate this requirement [94,95]. Ideally some applications, high spatial and temporal resolution must be achieved. This is the case for Single-Molecule or Single-Particle Tracking (SMT/SPT): approaches that focus on the tracking of single molecules or particles in cellular environments, to understand dynamic processes [96,97]. SPT has been used extensively over the last couple of decades to investigate many cell aspects, across a range of different targets and imaging modalities. One example is the Epidermal Growth Factor Receptor (EGFR), as reviewed by Clarke *et al.,* in 2019 [68]. Here, SPT is exploited to capture individual EGFR diffusion kinetics and their perceived level of confinement: differentiating between confined motion on the cell membrane or directed motion upon internalization or recycling. This is appealing for the design of targeted therapies as these measurements reveal functional information about the target molecules, such as interactions or association to specific cellular structures (e.g., lipid rafts or cytoskeleton). Within the SRM toolkit, techniques like PAINT and MINFLUX can be run in 'fixed mode' (maximizing spatial resolution) or 'live' or 'tracking mode' (maximizing temporal resolution, while still achieving a high spatial resolution). Live-PAINT [98] is one of the more recent SR-based approaches for SPT. Here, transient binding events between a fluorescently labelled probe and the target or interest are tracked. From this technique, kinetics of binding can be obtained together with the trajectories of the molecule of interest [72]. PAINT-based approaches enable the study of protein-membrane interactions or cell receptors in both fix and living cells [69,72,99], but also the tracking of target molecules in 3D [75] with a temporal resolution of tens of milliseconds. The development of novel probes beyond DNA have expanded the use of PAINT. For instance, to look at glycan-lectin interactions *a la* glyco-PAINT [50] (Fig. 3B), important to study immunity and for the development of vaccines. SRM has also been used to study viruses and their interactions with cells to aid the development of novel anti-viral therapies [100]. Finally, MINFLUX [71,73,91], which stands for "minimal photon fluxes', combines the strengths of STED and SMLM modalities to take SPT to an extreme. Here, the microscope locks on to a single molecule and actively readjusts XYZ to maintain focus. Sub-millisecond temporal resolution coupled with nanometer spatial precision yields an incredible in-depth view of the motion and path that a single molecule takes: maximizing both the temporal and spatial resolution achieved (Fig. 3C). MINFLUX has been combined with DNA-PAINT [101] to look at chromatin. It has also been used to follow a single kinesin-1 molecule on its intra-cellular walkabout [51], or to visualize nuclear pores in live cells and distribution of synaptic proteins in fixed cells [102]. MINFLUX is a shining exception that emphasizes the current trade-off between spatial resolution and temporal resolution.

Challenges and opportunities. Naturally, there are several limitations and restrictions of these methods. First, in PAINT methods non-specific binding of probes has to be carefully checked to validate the signals obtained. This is often a hurdle towards quantification as the low affinities of the probes result in a mixture of desired and undesired interactions. When going away from DNA, a main limitation is the availability of validated PAINT probes. Novel probes need to fulfill many requirements (low affinity, specificity, brightness, lack of toxicity) and their development is becoming an active field of research [85,103]. Here, image analysis is also crucial, where setting up effective workflows to extract diffusion parameters may have to be tailored to the molecule of interest. Also, there are restrictions on the data that can be generated in this manner. Typically, an image acquired with PAINT but also SMLM methods would yield tens of GBs of data. Therefore, acquisition, processing and storage are all aspects to be considered upfront. Finally, interpreting the data obtained and assigning its biological meaning (and its connection to the NP design) is far from trivial and often requires some pre-knowledge of the system at study. So far, the experimental expertise needed for SRMs coupled to their infrastructural requirements have limited a wide use of these techniques. However, this is rapidly changing. Many new commercially available setups as well as open-source microscopes designs have been reported in the last years. Kits for SRM-tailored labeling and sample preparations are now commercially available. All these developments promote the use of SRM among non-experts, reducing setup costs and improving their availability. SRM nearly passed its infancy, and we envision that with time, analogously to other new instrumental methods developed in the past, nanoscopy will be a standard technique in the arsenal of biologists and chemists.

2.3. *Understanding the material*

The next step towards a full understanding of nanomedicines is to understand the nanocarriers themselves. There is a plethora of analytical techniques to characterize nanomaterials [104]. Despite the efforts to standardize nanomaterial characterization [105], the optimal set of physicochemical properties to report is still an open question [106]. Regardless of this lack of standardization, properties such as size, morphology, shape, stability, and zeta-potential are routinely reported; and the importance to characterize nanoparticle composition and surface properties is recognized [105-107].

Nanoparticle size is often measured with light scattering techniques, most commonly Dynamic Light Scattering (DLS) [108]; while size at a single-nanoparticle level is often characterized by Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) [109]. DLS provides the effective size of the nanoparticle in solution, but it is limited to spherical particles [106]. It also provides an indication of colloidal stability due to its sensitivity to aggregation. However, the polydispersity index (PDI) derived from this measurement, fails to describe the heterogeneity in size of the sample; especially for highly dispersed particles or mixtures containing several populations. This heterogeneity can only be revealed with TEM and AFM [109], and intra-particle heterogeneities have been shown to impact *in vivo* behavior [110,111]. In addition to particle size, TEM and AFM can also resolve the carrier's morphology. However, the roughness of the sample preparation limits the study of nanomedicines under their 'native' conditions [45]. Super Resolution Microscopy (SRM) can overcome this limitation, providing structural information with minimal invasiveness [33], and therefore, suitable for characterization of fragile NPs in physiologically relevant environments. With recent developments on fluorescence labelling, quantification of ligand density [112], accessibility [61], orientation [113] and even protein corona formation [114] on the NPs' surface have been demonstrated by SRM. In this section, we discuss how SRM can shed light on nanocarrier's structure and properties at a single-nanoparticle level. We focus on SRM's capability to quantify single molecules [115], detect multiple targets [116], and complement other techniques (correlative imaging) [55].

Quantification. The presence, distribution and accessibility of functional ligands on the surface of the nanomedicine play a critical role in the cell uptake [117]. Quantitative PAINT (qPAINT) can quantify the number of ligands on individual NPs: information that is not accessible by other methods. Indeed, ligand quantification is often not carried out and the ligand quantity is inferred assuming that all the ligand introduced in the synthesis is on the NP surface or measured with very indirect and qualitative methods. Pioneered by Jungmann and coworkers [115], qPAINT relies on the predictable binding kinetics of short DNA oligos rather than the complex photo-physics of fluorophores. This strategy enables the calculation of the number of binding sites (n), using Eq. (1):

$$n = (K_{on} C_i \tau_d)^{-1} \quad (1)$$

where K_{on} is the association rate of the probe to the target, C_i the concentration of probe, and τ_d is the averaged dark time between binding events [115]. By labelling probes with DNA oligos, a multitude of functional ligands can be mapped and counted. For example, the number of antibodies on single silica nanoparticles by qPAINT [112]. Recently, Andrian *et al.,* has applied this principle to study polylactic polyglycolic acid-polyethylene glycol (PLGA-PEG) nanoparticles, which offers a versatile platform for the development of nanomedicines [61]. In this study, the accessibility of ligands on the NP surface is quantified by conjugating DNA docking strands to the maleimide motif at the end of the PLGA-PEG chain, a functional group normally used to conjugate functional ligands for targeting. Such quantitative characterization revealed the impact of PEG architecture on ligand accessibility (Fig. 4A). This work shows the potential use of qPAINT as a routine characterization for ligand quantification and availability on nanomedicines.

Multicolor. The surface of NPs is, in reality, more complex than a homogenous coverage of perfectly oriented ligands [118], as often illustrated in explanatory or schematic cartoons. Ligand quantification using SRM techniques such as DNA-PAINT, have revealed distinct distribution patterns on the surface of nanoparticles (*intra-particle heterogeneity*) and differences between particles within the same batch (*inter-particle heterogeneity*) [52]. This heterogeneity can be more dramatic, when more than one ligand is incorporated in the same platform to, for instance, exploit multivalency in targeting [119,120].These heterogeneities across NP surfaces will then impact the absorption of biomolecules on their surface, as shown by Feiner-Gracia *et al.*, [114]. When NPs enter in contact with biological fluids, adsorption of biomolecules on the NP surface occurs, forming the so-called biomolecular corona, which determines the biological identity and fate of the NP [121]. The composition of the molecular corona, and its evolution over time, depends on the route of administration, the patient, and the surface properties of the NP [30]. Therefore, characterization of NP surface properties and their 'biological ID', is pivotal when designing effective nanomedicines. To probe this complex landscape, multi-dimensional characterization is needed. The broad range of fluorophores and versatile fluorescent labelling strategy have enabled researchers to map

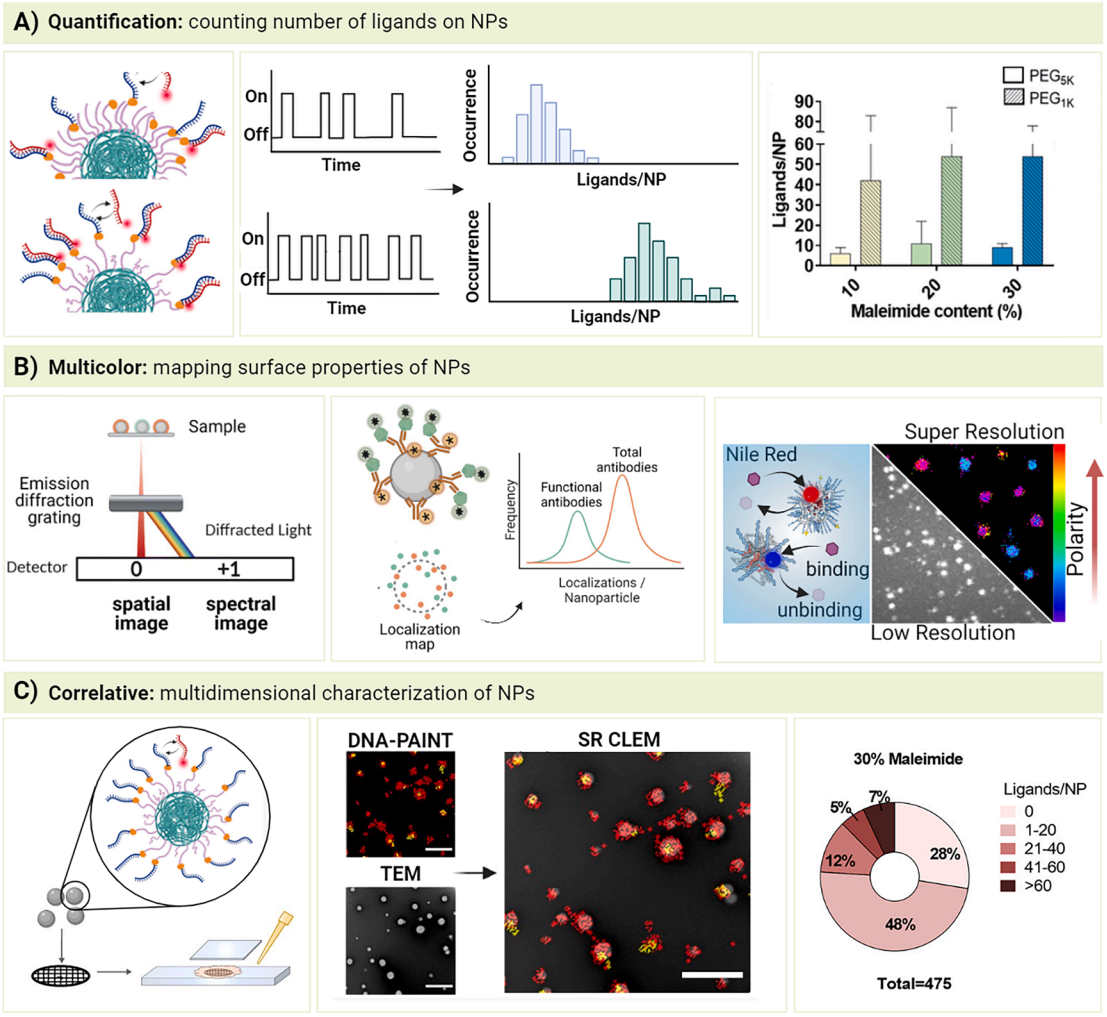

Fig. 4. Unravelling nanoparticle (NP) properties at the single-molecule resolution. A) Quantitative analysis of ligands on NP surface. Schematic cartoon showing two Polylactic polyglycolic acid-polyethylene glycol (PLGA-PEG) NPs with two different PEG lengths (1 kDa, 5kDa) and the accessibility of the ligand (maleimide) to the DNA-probe; the corresponding time traces and quantification of available ligands; comparison of ligands/NP when varying PEG length and maleimide content. Figures adapted from [61]. B) Multi-color Super-Resolution (SR) to explore the complex landscape of NP surface. Schematic representation of spectral setup; counting functional antibodies (with the antigen-binding fragment exposed); probing hydrophobicity of single-chain NPs using Nile red as a probe. Figures adapted from [53,54]. C) Multiparametric characterization of nanoparticles using Correlative Light E lectron Microscopy (CLEM). Preparation of NPs on CLEM substrate; representative images of DNA-Points Accumulation for Imaging in Nanoscale Topography (PAINT), Transmission Electron Microscopy (TEM) and overlay (SR CLEM); quantification of the number of ligands per NP, showing that almost 30% of the particles found in TEM did not have any ligand in DNA-PAINT. Figures adapted from [55].

specific and multiple ligands and proteins simultaneously on the nanoparticles surface [114,116]. A recent report demonstrated the visualization of protein corona composition on single nanoparticles using multicolor STED [116]. Here, the relative quantity of fluorescently labeled bovine serum album, immunoglobulin-G, and transferrin were estimated from the fluorescence intensity in different color channels. These studies focus on understanding nanoparticle surface interaction with blood components; essential to design nanoparticles that are administered intravenously. For non-parental administration, however, it is key to consider nanomedicine interaction with other fluids, such as mucus. Liu *et al.*, have recently reviewed the use of SRM to study nanoparticle interaction with mucus, driving nanocarrier design [122].

To fully exploit the power of multicolor detection, spectrally-resolved SRM has been developed to obtain the localization and spectrum of the target simultaneously. This technique only requires a simple modification of a TIRF microscope, by either placing a transmission grating in front of the camera or by splitting the emission optical path with a prism placed before the camera, obtaining localization from the 0-order image and the spectrum from the 1-order diffraction (Fig. 4B) [53,54]. Using such spectrally-resolved dSTORM, Archontakis, *et al.,* have demonstrated simultaneous mapping functional and total number of cetuximab antibodies (ABs) on the surface of silica NPs, by labelling all ABs with CF680 and detecting the exposed functional fragment (the antigen-binding regions) with a AF647-labelled EGFR probe (Fig. 4B) [53]. As with most targeting ligands, ABs need to be properly oriented to recognize the target [123], but also to avoid eliciting an undesired immune response [124]. More recently, Tholen *et al.,* [113] have proposed a qPAINT-based strategy to quantify antibody orientation on silica nanoparticles using engineered protein G and protein M probes that recognize crystallizable and antibody-binding fragments, respectively. In this study, the impact on AB orientation between different antibody conjugation strategies was assessed, as well as their impact on eliciting an immune response.

Spectrally-resolved SRM can also be used to probe local hydrophobicity of the material by using solvatochromic dyes, such as Nile red, which changes emission wavelength according to the local polarity [125]. This is especially attractive for the characterization of polymeric and lipid-based nanomedicines, where amphiphilic properties play an important role. Mapping the surface hydrophobicity has been demonstrated recently on single-chain polymeric nanoparticles using Nile red spectrally-resolved PAINT [54]. Here, the Nile red molecules transiently bind to the surface of the NPs, yielding a blinking signal to allow precise localization and variable emission wavelengths to unravel the local hydrophobicity (Fig. 4B). With the development of new probes for SRM, new properties like charge can be mapped, gaining a richer view of NP physicochemical properties. Current advances in probe development were recently reviewed elsewhere [85].

However, the multiparametric imaging based on spectral differences of labeling has a limited capacity, with a typical three-color setting. To expand the multiparametric capacity of the SRM, exchange-PAINT, a spectrally unlimited labelling strategy with DNA barcode was developed [66,126], as discussed previously. Here, the target molecules are labeled with short DNA strands with distinct sequences and imaged sequentially with orthogonal dye-labeled complimentary 'imager' DNA strands. This modality has been mainly exploited for cell characterization, as shown in section 2.2, but it can be applied to nanomaterials.

Towards multiparametric characterization. Recent studies have shown that the interaction of the NPs with the cell are affected not only by the number of ligands and receptors, but also by the morphology and mechanical properties of the nanomedicine [127]. Therefore, in order to gain a better understanding of the NP-cell interaction and to get a rational design of nanomedicine, multiparametric characterization is required. This multiparametric data can be acquired by combining the information provided by different analytical techniques on the same sample: an approach known as correlative imaging [128], which has also been used in biology to study cellular organization with ultrastructural detail [129]. TEM has been used to precisely evaluate the size and shape of NPs and even the surface functional ligands when combined with immunostaining [130], while AFM has been used to characterize the morphology of NPs and more uniquely their nanomechanical properties [131,132]. Fluorescence microscopy, especially SRM, complement these modalities by specifically targeting the functional sites on the NPs, offering quantitative analysis with high precision. Combining SRM with AFM and TEM to characterize the same sample enables correlating the functionality of the particles with their sizes and other parameters of interest. The challenges to combine two imaging modalities mainly lie in finding the compatible sample fixation protocols and substrates. Correlative characterization combining AFM with conventional fluorescence microscopy has been demonstrated to evaluate extracellular vesicles [132]. Andrian *et al.,* has shown the characterization of PLGA-PEG nanoparticles by Correlative Light and Electron Microscopy (CLEM). In this study, up to 30% of the polymeric particles displayed no functional ligands on their surface, hence "invisible" in SR microscopy [55], as shown in Fig. 4C. Such heterogeneity can only be revealed by CLEM, which demonstrates the importance of multiparametric characterization.

SRM in nanomedicine industry. One of the translational challenges of nanomedicine development is the scale-up manufacturing of these materials [29,133,134]. To ensure robust and reproducible preclinical and clinical studies (as well as later commercialization), large-batches with desired properties must be produced according to Good Manufacturing Practice (GMP), quality-by-design principles and in compliance with FDA/EMA guidelines [29,133-135].Colloidally stable nanomedicines often synthesized in a lab environment in small quantities are difficult to produce in large amounts [29]. SRM could be exploited not only to understand the fundamental aspects of nanomedicines, but also be implemented during the scale-up process to evaluate the manufacturing process and ensure that nanoparticles are produced with the desired physicochemical properties. Moreover, the adoption of single-nanoparticle characterization techniques has revealed heterogeneities between particles in the same batch; invisible to bulk-measurements. There is increasing evidence that these heterogeneities influence nanoparticle behavior and must be understood in order to design safe and efficient nanocarriers [107,118]. However, the slowness or low throughput of SRM can constitute a major challenge for their industrial implementation. A potential route to break this bottleneck may include technical developments to increase imaging speed, automation and the development of low affinity PAINT probes to eliminate the tedious chemical labeling of the materials. We will discuss some of these solutions in the following sections. Challenges for adoption of SRM as routine techniques for nanomaterial characterization in academic laboratories was already discussed by Dhiman *et al.*, [45].

2.4. Understanding cell-material interaction

2.4.1. Standard techniques for nanoparticle evaluation

After fully interrogating the diseased cell and the material in detail, their interplay constitutes the final missing element of the puzzle. It is becoming clearer that understanding cell-material interactions is fundamental for the design of safe and effective nanomedicines. As discussed earlier, NP performance will be determined by the ability of the NP to overcome biological barriers (including but not limited to circulation, clearance mechanisms, immune system, extravasation and tissue penetration) from the moment it is administered to the patient (usually through a systemic route) until it reaches the target [16,21,22,22]. In the discovery stage, the attention is mostly focus on the last barrier (the cell), and cell-material interactions are interrogated *in vitro* in terms of safety and efficacy.

Nanoparticle safety. Traditional methods to assess cytotoxicity include **a)** *dye exclusion assays*, such as trypan blue; **b)** *colorimetric metabolic assays*, such as tetrazolium salts including 3-(4,5-dimethyethiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT); **c)** *cell*

proliferation assays, such as the labelled nucleotide analog 5-bromo-2′-deoxyuridin) (brdU); **d)** *fluorometric cell viability assays*, such as the intracellular redox-based test Alamar Blue [136-138]; and **e)** *functional fluorescent dyes*, including inclusion and exclusion dyes (Calcein and propodium iodide (PI)), live/dead dyes, apoptotic markers (Anexin V) or mitochondrial dyes (JC-10, Mitotracker) [137]. Viability and metabolic assays are very accessible, inexpensive, standardized and they do not require complex technical expertise. However, the information derived from these tests is limited, since they fail to provide spatial and single-cell information: completely missing out heterogeneities, or any mechanistical information about how precisely the nanoparticle impacts the cell. It is a 'rough' estimation of toxicity. Moreover, NPs may interfere with these conventional assays and bias the results [139]. In contrast, functional dyes evaluated by fluorescence microscopy (epifluorescence, confocal) and/or flow cytometry capture a more detailed picture of cytotoxicity, by interrogating single cells, differentiating live from dead cells, and even identifying mechanisms of cell death, when, for example, using apoptotic markers or dyes that indicate organelle dysfunction. We will discuss how the use of functional fluorescent dyes and probes, in combination with temporally resolved observations of cellular structures and morphological changes, set the ground for advanced optical imaging to investigate the impact or activity of NPs in cells with unprecedented detail.

Nanoparticle fate and efficacy. Imaging is also used to assess NP efficacy. While the assay of choice is dependent on the actual purpose of the material, assays like cell toxicity, cell uptake, and co-localization with cellular compartments are commonly used to establish performance. The most common techniques exploited for this purpose are flow cytometry, confocal microscopy, and TEM [140]. While flow cytometry is considered to have a high-throughput, which allows to interrogate populations of cells in minutes, it lacks the spatial information provided by microscopies (unless combined with imaging, so-called flow cytometry imaging [141]). For that reason, it is primarily used for assessing semi-quantitatively the magnitude of uptake or internalization. Confocal microscopy and TEM provide the spatial resolution needed for co-localization studies. While confocal microscopy is not always capable of discerning single nanoparticles in proximity (<0.2 μm) due to the light diffraction limit, TEM, which uses a focused beam of electrons instead of light, can even resolve single nanoparticles with 'nanometric' detail. However, TEM's throughput is extremely low, due to the complexity of sample preparation and the technique itself, and it is limited to fixed samples. Overall, there is a need to develop and adopt advanced methods with a high-throughput, amenable to automation, that provide multiparametric data and high resolution. Recent developments in fluorescence imaging are filling this gap and will be crucial tools for nanoparticles discovery.

2.4.2. *Towards High-Content and high resolution*

High-Content Imaging (HCI) is the intersection between automated fluorescent imaging with automated image analysis in cell-based assays [142]. Initially developed in the field of toxicology to study the cytotoxic effects of drugs, and soon after applied for compound identification and cell phenotyping [143], it is now being expanded to study nanomedicines interactions with cells [144]. HCI assays are *high-throughput*, since their protocols are amiable for full automation, from sample preparation to data processing (Fig. 5). HCI is also characterized by being quantitative and multi-parametric (high-content): it is based on the quantification of different parameters encoding spatial, temporal, fluorescent and/or morphological information [143,144]. Quantification of multiple parameters relies on the use of algorithms that can distinguish cellular structures and define them as 'objects of interest' (in a process called segmentation) [144]. Different optical modalities, including epifluorescence and confocal, are compatible with HCI [142,143]. Epifluorescence was the first optical modality used by HCI and still the most exploited now. A main advantage of epifluorescence, when compared to confocal or SRM techniques, is its high acquisition speed, which allows the generation of big dataset of images in a fleeting time, and its lower optical toxicity [145], which enables time-resolved live-cell imaging to study dynamic cellular processes. Potentially, every fluorescent-based assay can be adapted to HCI, adding spatio-temporal information. However, epifluorescence is limited by spatial resolution and restricted to thin samples. As discussed previously, 'zooming in' to single molecule or even sub-molecule level is key to get a clearer picture of the material and the cell. In parallel to the development of more advanced *in vitro* 3D models, confocal HCI has become more popular to assess safety and toxicity of drug compounds in spheroids, organoids, or organ-on-chip systems. Emerging imaging modalities will allow to interrogate complex samples with high

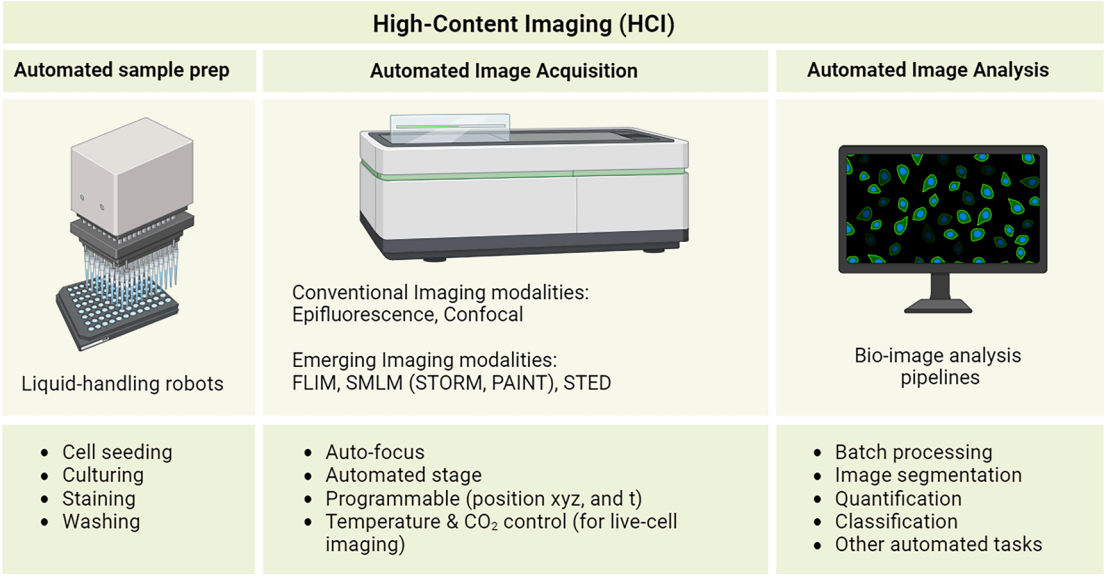

Fig. 5. High-Content Imaging (HCI) general workflow: A) automated sample preparation for multiple tasks: from cell seeding to staining. B) Automated image acquisition, including auto-focus, in several optical modalities: from epifluorescence to Super-Resolution (SR). C) Automated Image Analysis, incorporating different tasks to build a bio-image analysis pipeline. Abbreviations: Fluorescence-Lifetime Imaging Microscopy (FLIM), Single Molecule Localization Microscopy (SMLM), Stochastic Optical Reconstruction Microscopy (STORM), Points Accumulation for Imaging in Nanoscale Topography (PAINT), Stimulated Emission Depletion (STED).

resolution [146,147] and the acquisition of multiparametric data [148]. Here, we will discuss how HCI, focusing on epifluorescence and confocal modalities, has been used to study nanoparticle effects on cells focusing on safety and intracellular fate and how SRM techniques are complementary.

HCI for nanoparticle safety. Nuclear and membrane staining, live-dead functional probes, apoptotic indicators, proliferation markers, and a plethora of functional dyes and their combinations, can be used to study cytotoxicity effects of nanocarriers, their mechanisms, and their impact on cellular processes [149-151]. Anguissola *et al.,* proposed a multi-variate HCI assay to investigate sub-lethal cytotoxic effects of aminated polystyrene nanoparticles [149]. This assay was temporally resolved and monitored 7 parameters: cell count, nuclear size and intensity, mitochondrial depolarization, cytosolic calcium levels, lysosomal acidification, and plasma membrane integrity. A similar assay was exploited to evaluate the safety of a library of nanoparticles in different cell lines, suggesting the use of a panel of cells to unravel organ-specific toxicities [150]. Another HCI approach monitored changes in basal reactive oxygen species (ROS) levels simultaneously with mitochondrial depolarization and changes in morphology (size and circularity) [151]. Morphological readouts are indeed a powerful tool to investigate cellular perturbations. A prime example of this is the cell painting assay [152]. This approach uses 6 fluorescent dyes to extract ~ 1,500 features to build morphological profiles capable of detecting subtle changes in cell phenotype upon an external perturbation. These image-based phenotypic profiling methods [153,154] are mostly used to evaluate genetic (i.e., iRNA [155]) or chemical (i.e., small molecules [156,157]) perturbations. More recently Alijagic *et al.,* expanded this method to the study of metal-based NPs, by exploiting a cell painting approach in combination with metabolomics and lipidomics to study the safety of these NPs [56] (Fig. 6**A).** Cytotoxicity studies in 3D *in vitro* models are becoming more standard. Kelly *et al.,* applied a HCI multiparametric readout to assess the cytotoxicity of carboxylated vs. aminated polystyrene NPs in HepG2 spheroids [57]. In this study, individual cells were analyzed within the spheroid volume. The authors observed a heterogeneous distribution of dead cells within the spheroid and a high heterogeneity between spheroids. Thanks to the multiparametric nature of their readout, a phenomenon called *'lysosome swelling'* was observed upon amine-modified NP treatment, which correlated with the Propidium Iodide (PI) signal in dead cells. Lysosome swelling is a NP-related safety concern that is believed to occur when NPs reach the lysosome compartment but cannot be properly degraded, disrupting the organelle.

HCI for nanoparticle fate and efficacy. In most fluorescent-based nanoparticle trafficking studies, nanoparticle fate is estimated by co-localizing the carrier with different cellular compartments (endosomes, lysosomes, mitochondria, etc.). In non-phagocytic cells, NPs are believed to enter the cell primarily via active transport. Upon interaction with the cell membrane, NP internalization is triggered in vesicles,

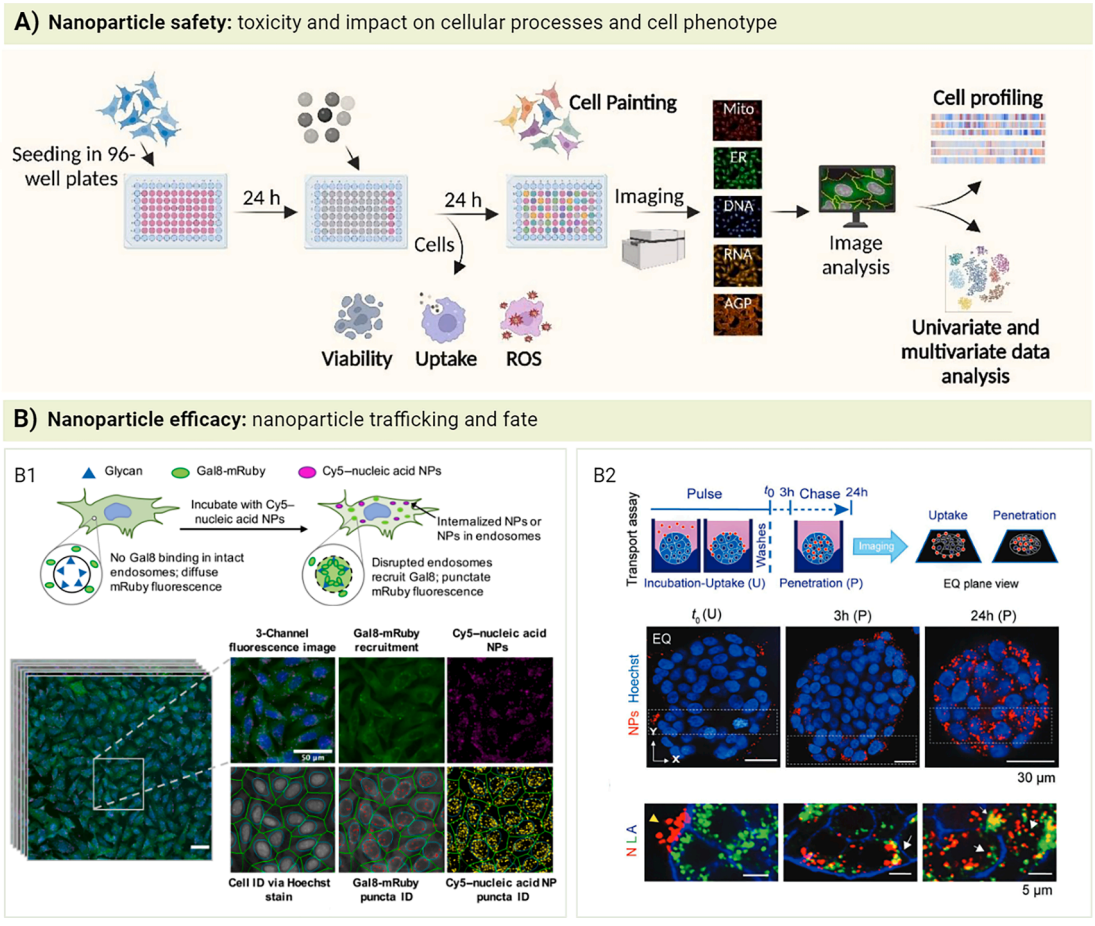

Fig. 6. High-Content Imaging (HCI) for nanomedicine evaluation. A) Nanoparticle safety. Workflow incorporating a Cell Painting strategy to study cytotoxicity of metal nanoparticles (NPs). Here, Mitochondria (Mito), Endoplasmic Reticulum (ER), DNA, RNA and Actin-Golgi-Plasma membrane (AGP) are fluorescently labelled, and more than 1,500 features are extracted from those 5 channels, to build the cell profiles. Adapted from [56]. B) Nanoparticle efficacy. B1) Evaluation of Cy5-labelled nucleic acid containing NPs in a mRuby-Gal8 cell line to visualize endosomal disruption. This image-based assay detects and quantifies Gal-8 puncta and Cy5 to estimate the degree of NP accumulation and endosomal escape. Adapted from [58]. B2) Nanoparticle fate evaluation in spheroids: Uptake and penetration. Automated image analysis pipeline to detect spheroids, segment cells and cellular structures and perform measurements on the spheroid's equatorial plane (EQ). Legend: nanoparticle (red, R), lysosomal markers (green, N), F-actin (blue, B). Adapted from [59].

known as endosomes. Once inside endosomal compartments, the NPs can follow different paths: they can accumulate, they can be re-excreted, or they can proceed to lysosomes and be degraded. It is believed that, for NPs to exert their function, they need to be able to escape this compartment and/or trigger their cargo release into the cell cytoplasm. This phenomenon is known in the field as endosomal escape, and can occur in a variety of (yet controversial) mechanisms [140]. Endosomal escape is assumed by the presence of nanoparticles on the cytosolic compartment, or by the lack of correlation with the endosomal compartments. Alternatively, endosomal escape can be observed using Calcein [156] or reporter cells [58,158-161]. Rui *et al.,* showed a direct method to visualize and monitor endosomal disruption in an HCI assay [58]**,** shown in (Fig. 6B1). In this work, the cell line model was genetically modified to express a recombinant Galectin-8 (Gal-8) protein. This sugar-binding protein binds to the glycans on the inner membrane of endosomes. Before endosomal disruption, the protein signal is diffused across the cytoplasm. Upon disruption, Gal-8 is recruited, and punctate fluorescence can be quantified. This assay was used to assess delivery efficacy of mRNA-loaded polymeric nanoparticles, and its predictive capability was later validated in a murine model. In co-localization or trafficking studies, switching from epifluorescence to confocal to gain in depth and resolution, results in a more accurate NP localization. In this modality, it is possible to resolve whether NPs are inside the cell or just bound to the surface, or if NPs are really inside a cellular compartment (e.g., endosome) or the fluorescence collected originated from another location above or below the focal plane. Cutrona and Simpson proposed a confocal-based HCI imaging method to measure NP penetration and uptake in 3D spheroids over time [59]. They describe a pipeline that identifies spheroids, locates their equatorial plane and segments individual cells and subcellular compartments **(**Fig. 6B2**).** After confirming co-localization of most of the NPs with lysosomal-associated membrane protein-1 (LAMP-1) positive vesicles (lysosomes and late endosomes), the authors tested a pilot iRNA assay to perturbate several protein targets involved in endosomal transport. They observed that, in control spheroids, NPs were distributed across the equatorial plane, but in siRAB7A-knockout cells (involved late-endosomal maturation and lysosome fusion) NP transport was hindered and NPs were only found at the periphery of the spheroid. These functional assays could also be applied to study nanomedicine performance in more complex biological models, like patient-derived organoids [162] or organ-on-chip models [163-165], which allows to recapitulate features from the tumor microenvironment. The use of these advanced biological models in nanomedicine research is still limited, but it will be slowly adopted, with further accessibility and standardization.

SRM for the rational design of targeted nanotherapeutics. However, confocal resolution may not be sufficient for some applications such as resolving cell ultrastructure or quantifying the number of particles in each compartment, since resolution is limited by the diffraction of light (~250 nm) and NPs in close proximity cannot be individually resolved. As mentioned earlier, the gold standard for observing individual nanoparticles in cell environments and investigating cellular ultrastructure is TEM. However, the family of SRM, introduced in section 2.1, can also break this diffraction limit and help us shine light onto cell-material interactions. In a recent review [34], Chen *et al.,* discussed the recent advances of SRMfor the study of nanomaterials and cell interactions: covering the three main families of SRM techniques (SIM, STED and SMLM) and their application on the study of NP internalization, intracellular fate and formation of protein corona. Focusing on the same family of techniques, Andrian *et al.,* discussed their use for quantifying NP endosomal escape [140]. Here, the authors highlight the potential of correlative methods in this arena. In a following work, Andrian *et al.,* demonstrated a CLEM approach combining STORM and cryo-TEM to study the intracellular trafficking of pathogens [166]; such method could be generalized to NPs. Few CLEM protocols have been proposed for the study of nano-cell interactions, partly because of their technical complexity and restricted accessibility. However, we expect a higher adoption of these techniques in the following years, since they hold a great potential to generate multiparametric data that cannot be accessed otherwise.. In short, for studies prior 2021, regarding the use of SRM (alone or CLEM) to study nano-cell interactions, we refer to these works [34,140]. More recently, SRM have been exploited directly for the rational design of targeted nanomedicines, correlating NP properties, such as ligand density, distribution and orientation with their biological function, or targeting ability [60,117]. Woythe *et al.*, [60] used dSTORM to quantify the number of functional targeting moieties on the surface of NPs and the expression of the targeted receptor in a panel of breast cancer cell lines; finding the optimal targeting regime for the given NP platform and target cell. Using the same SRM technique, the influence of valency, aptamer affinity and receptor density on targeting selectivity was studied [117]. Indeed, dSTORM is one of the preferred SRM modalities for studying NP-cell interactions [167-169]. However, dSTORM, like other SMLM techniques, is limited by its slowness and low throughput. Working on multiwell plates, automating acquisition and data analysis, as well as dealing with high volumes of data are new challenges emerging in this field. In the last few years, some efforts have been directed on adapting advanced and super-resolution techniques to a high-content format. On 2017, Beghin *et al.,* described a platform for automated HCI 3D dSTORM and DNA-PAINT in a 96-well plate [146]. More recently, in 2023, Barentine *et al.,* introduced a custom-build setup capable of acquiring 3D multicolor STORM in a high-throughput way [147]. Despite being developed separately, high-content imaging and super-resolution are starting to converge. These advances show that it is possible to consider the integration of high-throughput, high-content and super-resolution in the same workflow.

Beyond cultured cells. The use of optical imaging *in vivo* to evaluate nanoparticle safety and efficacy remains challenging [170,171]. With the exception of the zebrafish model [172,173], *in situ* visualization of nanoparticle distribution *in vivo* is performed by non-invasive imaging techniques such as Positron Emission Tomography (PET), Computerized Tomography (CT) or Magnetic Resonance Imaging (MRI)[174,175]. However, extensive effort is invested on developing optical modalities to image small animals, including near infrared (NIR) optical imaging [170] ultrasound imaging [176], or SRM [171]. However, these techniques are still in their infancy. They are heavily limited in tissue depth, they require complex equipment and advanced probes [170], and they are invasive [177]. Optical imaging can aid pre-clinical and clinical studies by evaluating *ex vivo* samples. In a clinical setting, SRM can be used to analyze patient samples from blood tests [178] to biopsies [179]. In this way, SRM has the potential to assist pathologists in understanding cancer pathogenesis, improving diagnosis, patient stratification and consequent evaluation of novel (nano)-therapeutic strategies.

2.5. *Machine learning (ML) in advanced microscopy*

These rapid advances in microscopy imaging have taken us into a new era of complexity, presenting researchers with increasingly intricate images and a high volume of multiparametric data that surpass the analytical capacity of the human mind within practical time frames. As a result, the aid of computers becomes indispensable, not only for the analysis and interpretation of these images, but also for their efficient acquisition, enabling researchers to extract valuable insights in a fraction of the time. ML is the ability of a computer algorithm to learn from experience, both in a supervised (when the ground truth is known) or unsupervised way [180]. In bioimage analysis, these algorithms can be trained to segment images into regions of interest, quantify different parameters, recognize patterns in the data, and even classify cells and predict their behavior over time [181,182]. Therefore, ML is a powerful tool that can aid different aspects of microscopy. Here, we focus on three core tasks: image acquisition optimization, annotation and processing (Fig. 7).

Image acquisition optimization. SRM can resolve biological

structures and materials with nanometric detail. However, the imaging time required is often long and can limit its practical applications. Both ML and deep learning, a subset of ML based on neural networks, can help optimize imaging time (Fig. 7**A**). There are several ways of doing this: by **(1)** automatically optimizing the acquisition parameters, such as automated focus, exposure, etc. [187,188]; **(2)** reconstructing a low-resolution image to a high-resolution one, exploiting neural networks trained on simulated or labeled SR images [62,189-191]; or **(3)** engineering the Point Spread Function (PSF). PSF is a mathematical representation of how a single point source of light or object is blurred or spread out in an optical image. Deep learning techniques are employed for PSF engineering to accurately determine an emitter's location [184,192], and localize densely overlapped emitters [193]. Alternatively, ML can guide the microscope into imaging in higher resolution when detecting relevant biological events [194].

Image annotation or ground truth generation. To effectively exploit neural networks for a variety of tasks, they need to be trained with labeled images. Generating ground truth requires accurately annotating the desired features on the images (Fig. 7B1). This is a tedious task, but fortunately, there is a plethora of online tools available to make manual annotation easier, such as MakeSense [195], Label Studio [196] CVAT [197] or CATMAID [198]. A comprehensive list of tools can be found on Github [199]. These tools streamline the process of labeling your own images and can significantly simplify and expedite the ground truth generation process, facilitating efficient training of neural networks. Alternative ground truth generation approaches include simulated labeled images and also crowdsourcing, where the task of manual annotation is distributed to a large pool of contributors, for example in platforms like Amazon Mechanical Turk [200]. However, crowdsourcing, despite its attractiveness, may not be the best option. A recent study compares the quality of crowdsourced annotations with professional annotations, and how the instructions given greatly affect the results, concluding that professional annotators outperform crowdsourcing [201].

Image processing. Microscopy is a powerful tool for visualizing structures and processes at the microscopic level. However, analyzing the large volumes of data can be challenging and time-consuming, especially when dealing with complex or heterogeneous samples. ML provides a solution to this problem by enabling automated analysis of microscopy images. Generally, image processing pipelines start with image restoration (Fig. 6B2), if they need to be deblurred [202] or denoised [185,203] and continue with image segmentation (Fig. 6B3) to identify the multiple objects of interest present in the image. For this purpose, instance segmentation is commonly used, to differentiate every individual object, in contrast to semantic segmentation, that groups objects by type (i.e., foreground vs. background). Some of the currently

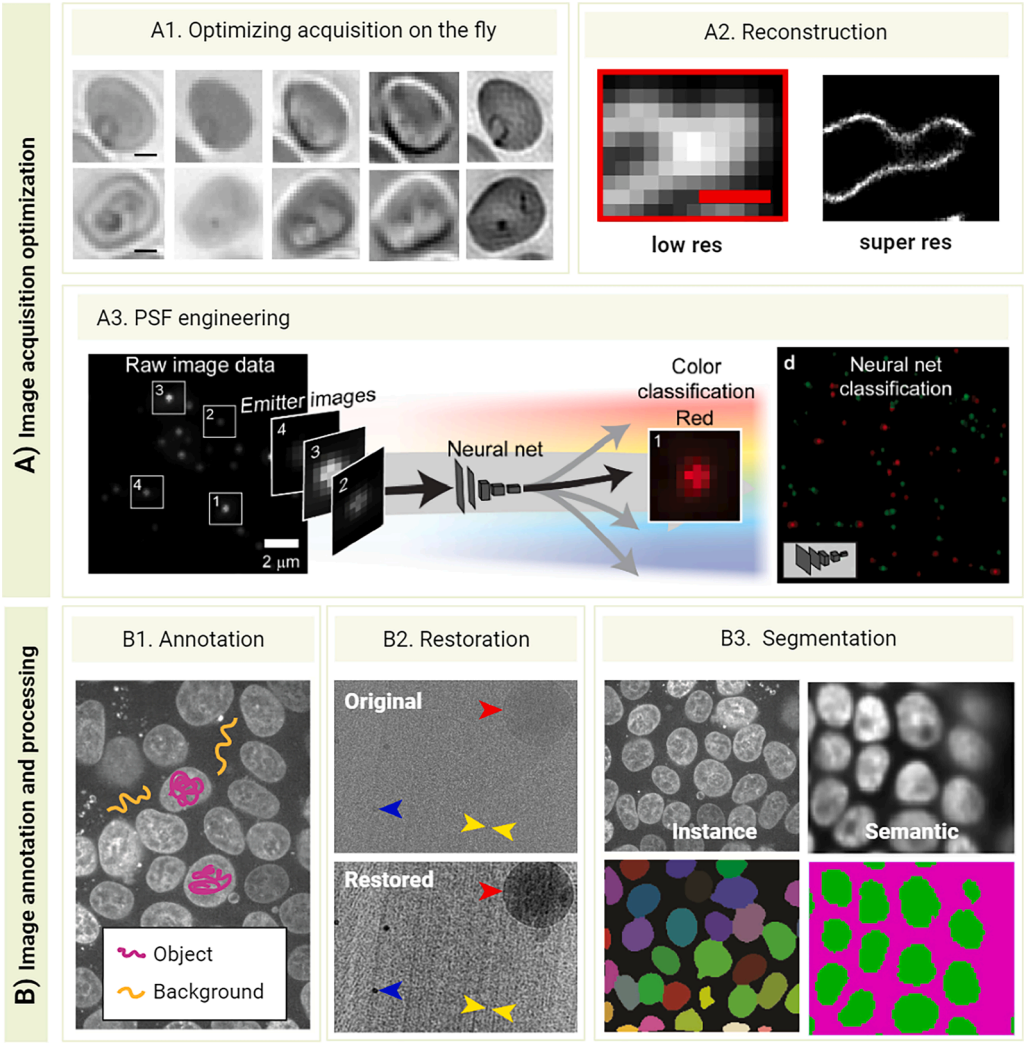

Fig. 7. Powering microscopy with Machine Learning (ML). A) Image acquisition optimization by A1) automatically optimizing the parameters (adapted from [183]); A2) reconstructing a super-resolution (SR) image from a low-resolution one A3) Point spread function (PSF) engineering (adapted from [62;184] © The Optical Society). B) Image annotation and processing: B1) annotations, B2) restoration, B3) segmentation. Adapted from [185,186].

available segmentation tools are listed in Table 1. Methods like DENOISEG [204] combine image restoration and segmentation, making image segmentation more efficient without needing high quality data, or preprocessing. Tools like CellProfiler (in combination with CellProfiler Analyst [205]), QuPath or Ilastik, include automatic classification and/or feature extraction of the objects detected. Finally, deep learning can automate the quantification and classification of microscopy images. Some of the tools listed in Table 1 use deep learning, such as StarDist or SplineDist, which have been pre-trained to recognize cellular morphologies. Many microscopy and image analysis groups have joint efforts into creating initiatives such as ZeroCostDL4Mic [206], a platform to use deep learning for microscopy image analysis without needing powerful computers or programming knowledge, or the bioimage zoo [207], which serves as an open access repository of ready-to-use algorithms for bio-image analysis. These models can be used in Fiji with the plug-in deepImageJ [208].

Responsible use of AI. The utilization of ML and deep learning algorithms offers significant advantages for bioimage acquisition and analysis. However, the inherent nature of these algorithms as black boxes raises concerns regarding replicability and potential inappropriate use. In a thought-provoking report, Laine *et al.*, describe how to properly validate and report procedures and results obtained through ML techniques [216]. When used responsibly, ML has shown to be a promising and indispensable tool in microscopy, facilitating the rapid development of advanced microscopy techniques. With further research and appropriate use, ML can continue to revolutionize the field of bioimage analysis, enabling new discoveries and advancements in the future.

Image processing in SMLM. In this review we emphasized how machine learning can aid the acquisition and processing of microscopy images in a broad sense. For SMLM, specifically, there are two crucial steps in image processing that have not been discussed, since they typically do not employ ML-based algorithms: (1) the Gaussian fit, for single-molecule localization and, (2) the post-fit localization analysis [45]. We refer to the following reviews for a comprehensive list of software available to process SMLM images and extract quantitative data from them [217,218]. Some of these software solutions provide additional data management, analysis and visualization tools [219,220]. The resulting data (e.g., carrier's size, aspect ratio, ligand density and distribution, intracellular location) can potentially serve as input for ML algorithms to find patterns, parameters or derive nanocarriers' structure–activity relationships.

Beyond image analysis: AI-powered nanomedicine development. In the past decade, ML algorithms have been used as predictive tools to accelerate drug discovery [221] and nanomedicine development [222-224]. ML models can be used to predict physicochemical properties of nanoparticles [225], optimize their biological response [37,226] or explore structure–activity relationships. We refer to the following reviews for ML-driven nanoparticle formulation and nanomedicine translation [39,222-224,227]. In most published work, the data fed into these algorithms comes from bulk measurements [228-233]. However, by using SMLM, we are able to characterize NPs at single-particle level [60,113,119] assessing their heterogeneity [55], an important structural parameter to understand nanoparticle performance [118]. We foresee that multi-parametric data extracted from super-resolution images can be a powerful fuel to aid the development of nanomedicines and understand nanocarrier heterogeneity and performance at the single-nanoparticle level.

Table 1
Main open-source image segmentation software packages.

Tool	Segmentation Type	Notes
EmbedSeg [63]	Instance	Both 2D and 3D images
nucleAIzer [209]	Instance	Parameter free nuclei detection
CellProfiler [186]	Instance	Feature extraction included and the option to upload your own pipeline
QuPath [210]	Semantic	Feature extraction included, for pathology images
LABKIT [211]	Semantic	Fiji plugin, both 2D or 3D images and videos
StarDist [212]	Instance	Detection of star-convex polygons
SplineDist [213]	Instance	Extension of StarDist for spline curves
Ilastik [214]	Instance/ Semantic	Workflows included, also as a Fiji plugin
DENOISEG [204]	Instance	Joint denoising and segmentation
Napari Assistant [215]	Instance/ Semantic	Napari plugin, 2D and 3D objects, other tasks included such as annotation.

3. Conclusion and future perspectives

It is an exciting moment for optical microscopy. Breaking the diffraction limit of light constituted a game changer in fluorescent microscopy for life sciences and nanomedicine research. Resolving cellular structures with nanometric precision and observing single nanoparticles is no longer restricted to EM or AFM. Advanced optical techniques or SRM can now resolve specific cellular structures and nanomaterials with high specificity, at a single-molecule or even sub-molecule level. Moreover, some SRM variants are also compatible with live-cell imaging and provide outstanding temporal resolution: allowing the study of dynamic cellular processes and mobile targets, such as receptors. This opens the possibility to study nanocarrier interactions with cellular receptors and structures, as well as their fate and impact on cells. Knowledge of expression of different therapeutic cellular targets, their relative abundance and behaviour (e.g., motility on cell membranes), may open new venues on understanding diseases; improving diagnosis, patient stratification and therapeutic strategy. Emerging imaging strategies (e.g., multiplexed PAINT, MINFLUX or CLEM) make it possible to interrogate a vast number of specific targets or properties on the same cell or material, reconstructing a more informative image of the object of study. The multi-parametric and functional data extracted typically from HCI-based assays provides mechanistical information on nanoparticle fate and impact on the cell. All this information can help our fundamental understanding of the carrier properties and unravel structure–activity relationships: essential for the rational design of safe and effective nanomedicines.

We emphasized the value of these techniques during the pre-clinical stage of nanoparticle development, but their adoption can critically impact other stages. In the nanomedicine industry, for instance, these techniques hold a great promise to be exploited during the scale-up manufacturing process: ensuring that large batches are produced with the same properties tested in the lab. Moreover, characterization of the carrier's properties and its homogeneity is pivotal to meeting the quality standards recommended by the regulatory agencies. Ligand density, distribution, accessibility at a single-nanoparticle level and their biological impact, will be key parameters to known to meet safe-by-design guidelines. This information is now accessible with SRM. For that reason, we believe that implementation of SRM characterization would help the understanding and translation of novel nanomedicines. We realize that SRM's slowness and low throughput can hamper their adoption in industrial settings. However, extensive efforts are already invested on formulating strategies to overcome this bottleneck, including: (1) Automation (or adaptation to high-throughput and high-content format), (2) development of novel PAINT low affinity probes, and (3) technical advances to increase imaging speed, analysis, and data interpretation.

Finally, we have discussed how machine learning can sustain the advancement of optical microscopy: from speeding and automating image acquisition, to improving data processing and their interpretation. In a wider perspective, ML models can also aid the rational development of nanomedicines, by identifying key parameters directly from the complex multi-parametric data generated, or by helping us understand structure–activity relationships. Altogether, advanced

optical techniques powered with ML can provide a powerful toolbox to help answering fundamental biological questions and developing optimized safe-by-design nanocarriers.

Declaration of generative AI in scientific writing

During the preparation of this work the authors used OpenAi (Chat GPT) in order to improve readability of long/wordy sentences. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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