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Scatter Enhanced Phase Contrast Microscopy for Discriminating Mechanisms of Active Nanoparticle Transport in Living Cells

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Abstract

Understanding the uptake and transport dynamics of engineered nanomaterials (ENMs) by mammalian cells is an important step in designing next-generation drug delivery systems. However, to track these materials and their cellular interactions, current studies often depend on surface-bound fluorescent labels which have the potential to alter native cellular recognition events. As a result, there is still a need to develop methods capable of monitoring ENM-cell interactions independent of surface modification. Addressing these concerns, here we show how Scatter Enhanced Phase Contrast (SEPC) microscopy can be extended to work as a generalized label-free approach for monitoring nanoparticle uptake and transport dynamics. To determine which materials can be studied using SEPC, we turn to Lorenz-Mie theory, predicting that individual particles down to ~35 nm can be observed. We confirm this experimentally, demonstrating that SEPC works for a variety of metal and metal oxides, including: Au, Ag, TiO₂, CeO₂, Al₂O₃, and Fe₂O₃ nanoparticles. We then demonstrate that SEPC microscopy can be used in a quantitative, time-dependent fashion to discriminate between distinct modes of active cellular transport, including intracellular transport and membrane assisted transport. Finally, we combine this technique with microcontact printing to normalize transport dynamics across multiple cells,

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Author Contributions

K.K.P. supervised the research. K.K.P., J.F.Z. and H.A.M.A. designed the study. J.F.Z. and H.A. M.A. performed the experiments. J.F.Z. and H.A.M.A. analyzed the data. G.P. and J.D. synthesized the ENMs and characterized their composition. B.M. and P.D. supervised the synthesis and characterization of the ENMs. All authors discussed the results and contributed to the writing of the final manuscript.

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Supplementary Materials

Additional supporting information is available free of charge on the ACS Publications website. This includes additional information on experimental methods for Hyperspectral imaging, Raman spectroscopy, oblique angle luminosity measurements, UV-Vis absorption spectroscopy, sample preparation for transmission electron microscopy, nanomaterial synthesis and dispersion, particle transport dynamics, Lorenz-Mie theory calculations, statistical analysis and signal-noise ratio measurements. This document also contains sixteen additional supporting figures as references in the text, four reference tables and captions for six supporting videos.

allowing for a careful study of ensemble TiO₂ nanoparticle uptake. This revealed three distinct regions of particle transport across the cell, indicating that membrane dynamics play an important role in regulating particle flow. By avoiding fluorescent labels, SEPC allows for a rational exploration of the surface properties of nanomaterials in their native state and their role in endocytosis and cellular transport.

Graphical Abstract

Keywords

Scatter Enhanced Phase Contrast; Endocytosis; Nanoparticle; Lorenz-Mie Theory; Nano-Bio Interface

Introduction

Engineered Nanomaterials (ENMs) have emerged as an important class of materials for use in biology and medicine, due to their unique size- and shape-dependent chemical, mechanical and physical properties.^{1,2} This allows ENMs to be introduced in a drug-like fashion, with potential applications as bio-orthogonal therapeutic devices and as vehicles for drug-delivery.^{3–6} However, as nanostructured materials have begun to see broader laboratory and commercial use, there is still a great need for understanding how these materials are internalized and distributed at cellular length scales. This is important for assessing the impact of existing ENM pollutants on human health and physiology, and for rationally controlling the mechanism of nanoparticle uptake. In this regard, nanomaterials have been reported to be internalized through a number of different receptor mediated pathways⁷, including clathrin^{8–10} and dynamin^{11–13} dependent endocytosis. However, predicting *a priori* if an ENM will be internalized, and by what mechanism, is still challenging as chemical composition, size, shape and surface properties all play a significant role in mediating ENM endocytosis.^{10,14,15}

In particular, isolating the role of surface modifications represents a significant challenge, as methods for studying ENM uptake and toxicity have traditionally depended on fluorescent markers for use in nanoparticle (NP) tracking.^{16–18} This can be problematic, as fluorescent labels can change the existing hydrodynamic radius and surface potential of a material, both of which have an impact NP endocytosis.^{15,19,20} Second, fluorescent labels can be toxic, making it difficult to distinguish between intrinsic NP cytotoxicity and the effects of surface coatings. Finally, the use of fluorescent labels can interfere with native protein corona formation and particle opsonization, further impacting downstream particle uptake.²⁰ While

this does not prohibit the study of inherently fluorescent materials, it creates a challenge in addressing the broader range of available NPs, such as metals and metal oxides which are among the most commonly produced nanomaterials.²¹ However, understanding these mechanisms of endocytosis and intracellular trafficking are essential for rationally engineering the delivery of NPs and in understanding the physiological impacts of existing materials. As a result, there is still a need to develop improved label-free methods capable of tracking ENMs in a real-time manner without the use of additional surface modification.

To address these concerns, here we show how Scatter Enhanced Phase Contrast (SEPC) can be used as a generalized approach for monitoring ENM endocytosis and particle transport. SEPC is an optical microscopy technique which allows for the simultaneous visualization of both cells and nanomaterials in a label-free manner. Previously reported in the context silicon nanowires,²² here we show how this method can be extended to smaller particles, whose largest dimensions are less than a 100 nm. This allows for the visualization of a range of metals and metal oxides ENMs. To understand the limits of this system, we use Lorenz-Mie theory as an accessible approach for understanding NP optical scattering, providing a theoretical framework for determining which particles can be tracked using SEPC. To validate this approach, we show that SEPC microscopy can be correlated to hyperspectral darkfield imaging and Raman spectroscopy, allowing for an independent assessment of ENM identity. We then demonstrate that SEPC can be used to track the real-time dynamics of ENM endocytosis across multiple particles compositions and cells types. Finally, combining this technique with microcontact printing, we demonstrate how SEPC can be used to study the normalized uptake of ENMs. Examining titanium oxide (TiO₂) NP endocytosis, we observe three distinct regions of transport, with particles exhibiting perinuclear clustering and spatially heterogeneous transport velocities. Collectively, this makes SEPC microscopy a powerful tool for understanding ENM-cell dynamics, providing insights into using nanomaterials as drug delivery systems and in understanding the environmental risk factors associated with existing ENMs.

SEPC Microscopy

SEPC allows for the simultaneous visualization of both cells and nanomaterials in a label-free manner by combining two traditional microscopy techniques: darkfield (DF) and phase contrast (PC) microscopy (Fig. 1a). Due to the difference in optical properties between NPs and cells, it is usually challenging to track both at the same time using a single approach. Inorganic NPs typically possess a high-refractive index, scattering light readily, making them easily discerned with DF microscopy. However, the sub-diffraction length scale of inorganic NPs makes them difficult to observe using brightfield techniques. Conversely, while adherent cells are difficult to visualize under DF because of their low refractive index, they can be tracked using brightfield techniques such as PC and differential interference contrast (DIC), both of which can provide high fidelity cellular imaging (Fig. S1). In traditional optical microscopy, both PC and DF condensers occupy the same position in the light path. This configuration limits the minimum achievable acquisition interval, as condenser changeover and re-calibration both require additional time, making it difficult to acquire real time images in this manner. Here, we show how SEPC microscopy can bridge

this gap by combining two-light sources to physically superimpose DF and PC images, giving SEPC microscopy the potential to track both cells and ENMs simultaneously.

To demonstrate this approach, gold nanoparticles (AuNPs) (18 nm diameter, Fig. S2, Table S2) were incubated with human umbilical vascular endothelial cells (HUVECs) and imaged under DF, PC and SEPC (Fig. 1b). As expected, this showed that NPs could be readily visualized under DF (Fig. 1b.i) while cells were apparent under PC (Fig. 1b.ii). Next, when combining each technique using SEPC, both cells and ENM could be distinctly recognized (Fig. 1b.iii). Using SEPC microscopy in this way means that ENMs can be placed in the context of the cell, in contrast to DF and PC techniques which primarily focus on these individual components separately. This allows for a better visualization of the interactions of ENMs with cellular systems. To quantify this, DF was used as an experimental control for NP location, and particle signal intensity was measured relative to the background intensity in each imaging mode. Using AuNPs as a control for signal intensity, SEPC showed a more than 20-fold increase in the signal-to-noise ratio as compared to PC microscopy alone (SEPC: 22.2 ± 2.4 , PC: 1.0 ± 0.8) (Fig. 1c). This indicates that using SEPC microscopy, AuNPs can be readily discerned from the background while still providing a clear visualization of the underlying cells. Additionally, this suggested that a variety of NPs could be studied using this approach. To further explore which types of nanomaterials can be visualized under SEPC microscopy, we used Lorenz-Mie (LM) theory to establish a framework for predicting the optical response of NPs.

Lorenz – Mie Theory Framework for Nanoparticle Optical Scattering

First reported by Gustav Mie in 1908 in the context of colloidal gold,²³ LM theory is an analytical approach to solving Maxwell's equations for the elastic scattering and absorbance electromagnetic plane waves. Here, we use LM theory as an accessible approach for understanding the optical scattering of ENMs. We note that numerical approaches, such as finite-difference time-domain methods (FDTD), can potentially provide a more accurate model by explicitly incorporating elements of cellular architecture.^{24–26} However, these approaches are often time intensive, both to implement and in terms of computational intensity. In contrast, LM theory has been widely reported for a number of different nanomaterials and geometric configurations, such as 1D wires or 2D sheets,²⁷ and is easily implemented using publicly available software (see Supporting Information). This allows LM theory the ability to rapidly address a variety of materials, making it a useful metric for initially determining if an ENM will be visible using SEPC microscopy. Under this model, the intensity of scattered light, $I(\lambda)$, is found relative to the illuminating light intensity, $S(\lambda)$, and the dimensionless scattering efficiency, $Q_{sca}(D, \lambda)$, (Fig. S3) such that,

$$I(\lambda) \propto S(\lambda) \cdot Q_{sca}(D, \lambda) \quad (1)$$

where D is the NP diameter, and λ is the wavelength of the illuminating light. As $Q_{sca}(D, \lambda)$ is proportional to the scattered light intensity, it offers a good metric for understanding how bright NPs will appear under DF and, therefore, SEPC microscopy.

Using LM theory we first calculated the wavelength and diameter dependent scattering and absorbance efficiencies, $Q_{\text{abs}}(D, \lambda)$ and $Q_{\text{sca}}(D, \lambda)$ respectively, of multiple ENMs (Fig. 2a), with reference values for the complex refractive index and the extinction coefficients obtained from the literature^{28–32} (Fig. S4). The metal and metal oxide particles studied showed strong spectral scattering in the ~300–400 nm range (Fig. 2a); with gold showing an additional peak in the visible regime, at 510 nm. In each case, an increase in particle size resulted in an increase in optical scattering intensity and a red-shift in maximal scattering intensity. The exception to this was SiO₂, which showed negligible scattering. To test the validity of LM theory for addressing these materials, the bulk absorbance of AuNPs was measured using UV-Vis spectroscopy (Fig. S5a), while the spectra of individual particles deposited onto a glass substrate was collected using hyperspectral imaging (Fig. 2b). We noted that an increase in particle size was predicted to lead to stronger optical scattering. To avoid biasing our sample toward brighter particles, dimmer background particles were selected for examination under hyperspectral imaging, as these were more likely to reflect the mean particle distribution as indicated by electron microscopy (Fig. S5b). In both bulk and single particle scattering, experimental measurements agreed with LM theory ($R^2 = 0.91$ & $R^2 = 0.86$, for bulk absorbance and individual scattering respectively) (Fig. 2b & Fig. S5b & c), showing a ~10 nm difference between the predicted and observed spectral peaks, which were likely the result of surface interactions with the underlying substrate. This approach was also used to examine additional ENMs, including Al₂O₃ and CeO₂ NPs (Fig. S5d & e), which showed similar matching between predicted and experimental values for individual particle scattering ($R^2=0.93$ and $R^2=0.90$, respectively). Collectively, these data suggest that LM theory is an appropriate model for studying NP scattering.

One key challenge in establishing which materials can be observed using SEPC microscopy is relating the predicted wavelength dependent scattering coefficients to the observable broadband optical scattering. This requires a constructing a weighted optical response which takes into account the spectrum of the illuminating lamp, the spectral sensitivity of the detector and the inherent interference of the internal optics. To achieve this, we constructed a normalized wavelength independent scattering coefficient, $Q_{\text{nsc}}(D)$ (Fig. S4), using a modified approach for reduction in dimensionality (see Supporting Information for details), as reported previously for silicon nanowires.³³ This allows for a more direct comparison between the predicted optical scattering and the observed intensity measurements under DF. Using this approach, we calculated $Q_{\text{nsc}}(D)$ for a library of metal and metal oxide NPs (Fig. 2c). Of the materials surveyed, Ag and Au showed the strongest predicted scattering, while SiO₂ exhibited a negligible response. As this approach in dimensional reduction has not been previously reported in the context of these materials, we wanted to validate that this approach offers reasonable experimental predictions. To test this, AuNPs of varying diameters were imaged using DF microscopy under matched optical conditions (0.5 – 3 second exposures, 50% illuminating power) (Fig S6). The resulting scattering intensities revealed a characteristic curve (Fig. S7e), which matched the trend predicted using the normalized scattering coefficients, $Q_{\text{nsc}}(D)$ (Fig. 2d). This suggested the validity of this approach. Additionally, this allowed for a direct comparison between the predicted and experimentally observed optical scattering, which in turn can be used to establish a threshold scattering intensity.

For NPs to be detected under SEPC, they need to have a higher scattering intensity than the surrounding medium. As adherent cells also scatter a small amount of light, this sets a natural detection threshold, below which it is difficult to distinguish individual particles from the surrounding cell. To determine this detection threshold, AuNPs of varying diameter and HUVECs containing no NPs, were imaged under DF using the same optical conditions (Fig. S7a–c). In each case, intensity measurements were then taken as a function of exposure time, with both cells and AuNPs showing a linear response in the relative photon counts (Fig. S7d). This allowed us to establish a detection threshold for particle scattering relative to the cells, by enabling a direct comparison between the calculated Q_{nsca} and the corresponding scatter intensity measurement. This yielded normalized scattering coefficients of ~ 0.010 and ~ 1.35 for the cell body and the perinuclear regions, respectively. These corresponded to AuNP diameters of 35 nm and 65 nm, respectively (Fig. 2d), suggesting that AuNPs with these diameters or larger can be distinguished within the cell. However, these sizes are material dependent. For instance, we would not expect SiO₂ particles to be distinguishable from the cell body until ~ 80 nm in diameter because of their poor scattering coefficients. Additionally, even at 150 nm in diameter the scattering of SiO₂ particles ($Q_{nsca}=0.125$) would be below the level of the perinuclear region. Overall, this creates a theoretical framework for understanding which materials can be tracked using SEPC, as the $Q_{nsca}(D)$ of many materials can readily be determined using LM theory. Finally, we note that while LM theory is most accurate in the case of spherical particles, $Q_{nsca}(D)$ can also be determined for intermediate sized materials, such as rods and discs, using an FDTD approach, allowing these results to be extended further to more complex geometries.

Confirming ENM Identity

To probe this framework and demonstrate that SEPC microscopy can be applied to a broader range of nanomaterials, a group of metal and metal oxide NPs of varying size and composition were imaged, including: Au (18 nm) (Fig. 1b), CeO₂ (35 nm), Al₂O₃ (28 nm), Fe₂O₃ (10 & 109 nm diameter), Ag (22 nm), TiO₂ (29 nm) and SiO₂ (15 nm) NPs (Fig. S8). These materials were selected as they are among the most commonly produced nanomaterials in a commercial setting^{34,35}, making them of interest from an environmental exposure perspective. Nanomaterials were synthesized and prepared as previously reported.^{36–39} (for a summary of ENMs used here, see Table S2). Additionally, a control sample containing no nanomaterials, was imaged. For those samples containing ENMs, NP suspensions (10 µg/mL) were incubated with HUVECs and imaged using both PC and SEPC microscopy. Among the materials surveyed using LM theory, Ag and Au showed the strongest predicted optical scattering, followed by Al₂O₃, TiO₂, Fe₂O₃ and CeO₂. SiO₂ showed an approximately two order of magnitude reduction in scattering efficiency as compared to other ENMs of a similar diameter (Fig. 2c). This matched our experimental findings, as all ENMs surveyed could be detected using SEPC, with the exception of the negative control and those samples exposed to SiO₂, both of which showed negligible scattering (Fig. S8). This suggests that SEPC is amenable to detecting a wide range of material compositions and particle sizes. We note however that many of the NPs observed this way were nominally below the detection threshold due to size considerations. As these materials could still be observed, this suggests that NPs were potentially clustering towards aggregates with larger effective cross sections and increased optical scattering. This is

consistent with previous findings, which indicate that NP endocytosis can lead to particle aggregation.⁴⁰

To study this aggregation phenomenon and confirm the identity of the NPs, hyperspectral imaging and Raman microscopy techniques were used to measure the spectral response of individual particle clusters (Fig. 3). While SEPC microscopy is limited in its ability to distinguish multiple nanomaterials from one another, hyperspectral imaging and Raman microscopy can be used to achieve this result, although these systems are difficult to use in a real-time, non-disruptive manner. In each case, ENMs were incubated with HUVECs and imaged using SEPC to identify potential particles of interest. Next, micrographs from the same region were obtained using hyperspectral imaging and Raman microscopy, allowing for a cross-comparison between microscopy techniques. For hyperspectral imaging, AuNPs were selected due to their large characteristic spectral peak in the visible range (Fig. 3a), distinguishing them from other metal and metal oxide NPs which predominately absorb in the ultraviolet region. For reference, an external control containing no cells was also imaged, measuring the spectra of multiple particles to obtain an average spectrum, with images smoothed to reduce shot noise ($n = 45$). For those particles incubated with HUVECs and observed under SEPC, the spectral response matched the external control, and with the predicted ~ 520 nm scattering peak (Fig. 3a.v). This confirmed the presence of AuNPs, suggesting the fidelity of SEPC for tracking ENM-cell interactions. Additionally, we noted that many of the experimentally observed samples showed a significant red-shift and spectral broadening—reinforcing the idea that particle aggregation was occurring (Fig. S9). The identity of the NPs was further confirmed using Raman microscopy. For these experiments, TiO_2 NPs were selected due to their distinct Raman spectra. For both cellular and reference samples containing NPs, a strong 147 cm^{-1} peak was observed (Fig. 3b), which is consistent with the previously reported E_g peak of anatase phase titanium oxide.⁴¹ Additionally, this peak was absent from the rest of the cytoplasm and from a blank glass control, containing no cells or NPs. Taken together, these results suggest that the objects visualized under SEPC are the desired ENMs, further indicating that they can be monitored to discern their uptake behavior.

SEPC microscopy can be used to track real-time particle dynamics, allowing researchers to discriminate between different modes of active cellular transport and ENM uptake (Fig. 4). For this study, HUVECs cultured on glass coverslips were exposed to NPs in Tyrode's buffer solution, allowing NPs to deposit onto the cells and the glass substrate, before being swapped into fresh buffer solution. ENM dynamics were then monitored under SEPC for two hours, with images acquired at two-four second intervals. To quantify particle transport, we tracked the particle's path, velocity and mean squared displacement (MSD), where MSD is the average distance that a particle travels as a function of lag time, given by the following:

$$MSD = \langle \Delta r^2 \rangle = D\tau^\alpha \quad (2)$$

with r , D , τ and α representing the particle's displacement, diffusion coefficient, observed lag period, and diffusivity exponent respectively. Measuring the diffusivity exponent, α ,

provides insight into the mode of particle transport, with different values indicating restricted diffusion ($\alpha < 1$), Brownian motion ($\alpha = 1$) and active particle transport ($\alpha > 1$). To measure α , a log-log plot of the MSD versus τ was fit using a linear least squares model, with the slope providing the α value (Fig. S10f). To understand the real-time particle dynamics, transport trajectories were segmented using a rolling-time frame analysis (1–3 minutes). Rather than providing a single α value for the entire particle trajectory, this allowed for changes in the particle transport characteristics to be monitored, as particles often shows intermittent periods of stagnation, and quick linear transport, providing a deeper insight into the temporal dynamics.

Tracking the diffusivity exponent, α , revealed several modes of ENM transport. In the case of intracellular transport, we observe short ‘burst-like’ periods as have been previously described⁴² (Fig. 4a)(Video S1). These were marked by high particle velocities (~200 nm/s in the present case), active diffusivity coefficients ($\alpha > 1$), and linear particle trajectories, with minimal movement of the cell membrane. This type of transport was typically brief relative to the entire acquisition period, lasting ~5 min, and in general showed an overall trajectory towards the perinuclear region. We note that this type of behavior was more consistent with motor-assisted vesicular transport, over cytoplasmic streaming, as the particles displayed bidirectional movement, back-tracking along their own path (Fig. 4a.v). This competition between anterograde and retrograde motion is indicative of assisted transport, suggesting the participation of kinesin and dynein motor proteins.⁴³ This type of transport was in contrast to other modes, such as membrane-assisted transport and particle uptake, which showed overall unidirectional motion.

In the case of membrane assisted transport, particles are carried along by the cell as the membrane deforms (Fig. 4b)(Video S2). Using SEPC, we can follow these deformations, allowing us to monitor the cell-ENM interactions simultaneously. This would be difficult to achieve using other realtime tracking methods, such as DF or PC, as these would miss the nanoparticle or membrane component respectively. In addition to motor protein assisted transport, movement by the cell membrane is also a form of “active transport” ($\alpha > 1$) as it requires energetic input from the cell. In contrast to other forms of intracellular transport, this mode shows significant membrane deformation and a lower maximum velocity; ~40–60 nm/s in the present case (Fig. 4b.iii). This is consistent with the slower speeds of cell motility, as compared to vesicular transport. Additionally, the time span of membrane assisted transport was more protracted, occurring over an extended duration (~12 min in the present case). Similarly, no ‘burst-like’ activity or bidirectional motion was observed in this case, with the particles instead favoring a more constant gradient of motion, progressing steadily in a single direction (Fig. 4b.iv & v). This type of motion is also consistent with motility directed transport, as cells change direction on a different timescale than intracellular motor assisted transport.

Finally, we can also compare both of these types of transport to the initial NP uptake event (Fig. 4c) (Video S3). In this case, the NP starts external to the cell, with no initial overlap. The membrane can then be seen to extend out and make contact with the particle (Fig. 4c.ii), at which time the particle goes from a stationary to a mobile state. Putting on a brief burst of speed, the particle is brought into the contact with the cell body, where it then becomes

relatively quiescent, showing restricted diffusion ($\alpha < 1$); presumed to be awaiting motor assisted transport to the perinuclear region. Similar to the previously discussed modes of transport, this event also shows periods of active cellular transport ($\alpha > 1$) (Fig. 4c.iii), however it displayed relatively low velocities compared to either membrane or motor assisted transport ($\sim 10\text{--}20$ nm/s). Additionally, similar to the membrane assisted transport case, the NPs showed relatively linear motion, with a significant amount of membrane deformation.

The NPs used in the previous example were aluminum oxide; however, this type of tracking can also be extended to work with a range of material compositions and cell types. For instance, here we also demonstrate that the active transport of cerium oxide NPs can be tracked in human aortic smooth muscle cells (HASMCs), showing similar transport dynamics to aluminum oxide (Fig. S10) (Video S4). This suggests that SEPC microscopy is applicable to a range of cell types, although in the case of non-adherent spherical cells, large membrane curvatures may make it difficult to distinguish particles from the background scattering. Additionally, we note that while real-time single particle dynamics have been studied for gold,⁴⁰ and ensemble studies have been performed for nanoceria^{44–46} and alumina^{47,48} NPs, this is one of the first reported case of single NP active transport for both nanoceria and alumina. In addition to multiple cell types, this technique can also be used to track a large number of NP all at once. Here we show the simultaneous tracking of ~ 280 TiO_2 NPs (Fig. S11) co-incubated with HUVECs, undergoing a variety of transport processes, including both initial NP uptake (Fig. S11b) and membrane assisted transport (Fig. S11c), with peak speeds of ~ 40 nm/s and ~ 250 nm/s respectively. Taken together, this suggests that SEPC can be used to explore a variety of ENM-cell interactions.

Composite Nanoparticle Dynamics

During multi-particle tracking for pleomorphic cells we noted that there was a spatial heterogeneity in particle transport dynamics within a single cell, where particles could exhibit dissimilar velocities even after similar particle exposure times. This suggests that different regions of the cell may confer distinct transport dynamics. In the pleomorphic case, this effect can be attributed to the influence of cell migration and membrane deformation as revealed by SEPC microscopy, with the lamellipodia traveling at different rates at each end of the cell. However, this makes it difficult to decouple innate transport behavior from cell motility. Even when the membrane remains relatively stationary, variability in membrane morphology means that particles will transverse different distances to reach the perinuclear region. This makes it challenging to normalize transport behavior across multiple cells and multiple NPs. To account for this, we combined SEPC microscopy with microcontact printing to form reproducible cell morphologies. Microcontact printing uses micromolded polydimethylsiloxane (PDMS) stamps to selectively deposit extracellular matrix proteins, forming controllable fibronectin islands (Fig. 5a). As cells will preferentially bind to the deposited matrix proteins, this can be used to guide cell shape giving rise to reproducible cell morphologies. Here, we microcontact printed fibronectin islands ($2000\text{ }\mu\text{m}^2$ area) onto PDMS coated glass coverslips, forming arrays of circular HUVECs (Fig. S12). The ability to create reproducible cell islands allows us to normalize NP transport across multiple cells, providing robust data on the temporal dynamics of particle uptake. For this study, we choose

to focus on TiO₂ NP uptake, as their brightness and high refractive index make them one of the most abundantly produced nanomaterials, seeing wide spread use in inks, pharmaceuticals, food products and cosmetics⁴⁹. Recently there has been concern over the ability of intravenous TiO₂ particles to transverse biological barriers, showing toxic effects in the brain and liver of developing mice models^{50,51}. Understanding the temporal dynamics of nanoparticle uptake can offer potential benefits in controlling and preventing deleterious particle exposure pathways.

Particle uptake was monitored using single cell islands exposed to 10 µg/mL of 100 nm TiO₂ NPs for 10 minutes, allowing NPs to deposit onto the arrayed islands. After being exchanged into fresh media, samples were then imaged using SEPC microscopy at 20 minute intervals. In a representative SEPC micrograph of a single cell island (Fig. 5a), we observe that particles go from being randomly distributed to centrally concentrated over the span of ~50 minutes. To quantify this behavior, we superimposed cell micrographs to form a single composite image of NP uptake (n = 3 samples, with 24, 32, and 38 cell islands composited for 10, 30, and 50 minutes respectively) (Fig. 5b). The location of the nucleus was normalized by centrally rotating each micrograph, such that the centroids of the nuclei were registered to the same angle (top-middle, Fig. S13). Composite images were then converted to polar coordinates and the average intensity was taken as a function of distance from the center of the cell. This allowed for a quantification of the average particle position as a function of time (Fig. 5b). Using this approach, we observed that particles began as evenly distributed across the cell, with a maximal intensity at a radius of 17.5 µm from the center. After 30 and 50 minutes, this was reduced to 6.8 and 4.0 µm respectively. This indicated that the bulk of nanoparticle transport occurs within the first 30 minutes of exposure, but that particles showed continued clustering after this point. Additionally, this further supported the idea of a spatial heterogeneity in transport dynamics, as particles showed greater clustering during the initial period, suggesting that particles move faster near the edge of the cell and slow down as they approach the nucleus.

To test this behavior, we measured the ensemble dynamics of TiO₂ NPs during particle uptake. Here, multiple cell islands were monitored in real time manner for a period of fifty minutes at four second intervals using SEPC microscopy (n=3 samples, 12 total cells). Particle trajectories were then extracted as previously described for individual cells (Fig. S14a) (Video S5), and composite dynamic maps were constructed by binning the average velocity (Fig. 5d) and diffusivity coefficients (Fig. S14b) of each particle in a region. Using this composited approach, we were able to construct a normalized vector map (Video S6) over a plurality of the cell (46.4% area coverage), avoiding potential conflicts of particle overloading and NP indistinguishability in tracking.

Mapping the cell in this manner, allowed for a clear visualization of particle dynamics across the cell, revealing three potential regions of interest. First, the edge of the cell appeared to have the highest particle velocities, with the cytosol exhibiting intermediate velocities, and the perinuclear region showing the lowest speeds. To quantify this behavior the average velocity and diffusion coefficient of the NPs was determined as a function of distance from the centroid (Fig. 5d). This showed that particles exhibited a steady decline in transport velocity as they approached the nucleus, with an average velocity of 54 ± 14 nm/s at the outer

edges of the cell, down to 6.9 ± 1.6 nm/s in the center. Interestingly, while velocities showed a steady decline, the diffusivity exponent, α , revealed three distinct phases of transport in the cell, with discrete steps beginning at ~ 7 μ m and ~ 17 μ m from the center of the cell. We note that these values align well with the peak clustering distances observed previously (Fig. 5b). To test whether these regions of particle transport were an artifact of velocity binning, we also examined individual particle trajectories. Similar to the ensemble case, we observed that particles undergo three distinct phases of transport in the cell (Fig S15), transitioning from ~ 40 nm/s at the edge of the cell down to a basal velocity of ~ 1.2 nm/s. The diffusivity coefficient also showed three distinct regions of transport as observed in the ensemble case. Collectively, this indicated that there are spatial heterogeneities in NP transport dynamics across the cell. To understand this behavior, we looked at the cytoskeletal organization and small perturbations in cellular membrane morphology during ENM exposure.

Because actin filaments serve as an important scaffolding for intracellular vesicle transport, we hypothesized that for particles to cluster in the perinuclear region, actin filaments would also need to converge. Upon fluorescently labeling the actin network, this is indeed what we observe, with labeled cell islands exhibiting radially aligned actin filaments that coincide at a central position (Fig. 5f). Additionally, upon comparing the radial projection of the actin filaments and to the composite NP velocities (Fig. S14c), we found that both exhibit similar linear alignments, suggesting that the actin filaments helped guide particle clustering in the perinuclear region. We note that such radially aligned chiral actin networks have previously been reported for micropatterned cells,⁵² indicating that this is a commonly found motif in cellular architecture. This clustering of the actin filaments suggests a potential explanation for the drop in transport velocities near the perinuclear region. As particles and actin filaments become more densely packed, this results in crowding in the cellular microenvironment, which can lead to a reduction in transport speeds^{53,54}. Additionally, as particles near the perinuclear region they can become trapped in lysosomes⁵⁵, resulting in further restricted diffusion. Taken together, this suggests a mechanism for the reduction in NP transport velocities near the perinuclear region.

To probe the mechanism of increased particle transport near the edge of the cell, we used SEPC microscopy to measure local cellular membrane deformations. As lamellipodia protrusions and retractions have been shown to regulate retrograde flows⁵⁶, we reasoned that this process could also be impacting NP transport. While microcontact printing results in normalized cell shapes, it does so by forming focal adhesion complexes with the underlying extracellular matrix proteins⁵⁷. As a result, some cells are still able to extend their lamellipodia past the fibronectin island by expanding their actin network, creating small changes in local membrane curvature. To measure these deviations in the lamellipodia, we used SEPC microscopy which revealed an ~ 10 μ m region of membrane instability (Fig. S16). This corresponded well with the observed region of increased activity near the edge of the cell. Additionally, NPs exhibited similar transport velocities compared to those reported for membrane protrusion (54 ± 14 nm/s for nanoparticle compared to ~ 50 nm/s for retrograde flow)⁵⁶. Collectively, this suggests that NP transport is regulated by membrane dynamics in this region of the cell.

Overall, this highlights the important role that membrane dynamics in nanoparticle transport. For both pleomorphic and microcontact printed cells, we observed membrane dependent effects. In the pleomorphic case, we observed distinct differences between membrane-assisted transport and other modes of active transport, while for microcontact printed cells we observed that membrane instabilities can potentiate retrograde flow. The ability of SEPC microscopy to track both NPs and cells simultaneously allowed us to resolve these behaviors, which can be difficult to achieve when tracking NPs alone. As a result, this work suggests that membrane dynamics play a larger role in nanoparticle uptake and transport than has been previously reported.

Conclusion

Here, we have shown that SEPC microscopy can be used as a generalized approach for studying label-free nanomaterial uptake—offering insights into spatial heterogeneity in NP transport. In the present study, we have limited our approach to metals and metal oxides as these are among the most commonly used materials in a commercial setting. However, we also believe that this technique has potential implications for a much wider range of emerging materials, such as polymeric materials, which are routinely studied using optical scattering approaches. To supplement this, we have shown that Lorenz-Mie theory can be used to predict the optical scattering properties of nanoparticles, providing a framework for understanding which materials can be tracked using SEPC microscopy. We were able to achieve this by introducing a normalized wavelength independent scattering coefficient, $Q_{nscd}(D)$, which allowed us to directly relate predicted scattering coefficients to experimentally observed optical intensities. This indicated that SEPC could be used to distinguish nanoparticles down to ~35 nm in size. We then used SEPC microscopy to track different modes of active transport in both pleomorphic and microcontact printed cells. In each case, the ability of SEPC microscopy to simultaneously track both nanoparticles and cell membranes offered key insights into nanoparticle uptake dynamics. As a label-free approach, SEPC has the potential to address several key challenges in the field. First, SEPC microscopy can be used to explore the impact of surface modification on nanoparticle uptake. While this has been studied for labeled materials,⁵⁸ it has previously been difficult to compare these results to label-free control. In this way, SEPC allows for a rational exploration of the surface properties of a nanomaterial and their role in endocytosis and cellular transport. Second, this technique has the potential to offer new insights into endosomal escape and nanomaterial exocytosis pathways. Research to date has primarily focused on initial nanoparticle uptake mechanisms,^{40,59} however, little known about the long term fate of intracellular particles.⁵⁹ This is in part due to a reliance on fluorescent labels for nanoparticle tracking, which can break down in the harsh environment of the lysosome. This limits the lifetime of fluorescence-based uptake and distribution studies. Using SEPC microscopy, it should be possible to continue tracking nanoparticle activity after this critical juncture, potentially offering new insight into these less understood pathways. Collectively, this suggests that SEPC has important implications for designing next-generation nanoparticle-based drug delivery systems and in better understanding the cellular interactions of existing nanomaterials.

Methods

Cell Culture

HUVECs culture was maintained under sterile conditions, at 37° C and 5% CO₂, using Medium 200 (Gibco) with the corresponding low serum endothelial growth supplement (LSGS, Thermo Fischer Scientific) which contains, fetal bovine serum (2% v/v), hydrocortisone (1 µg/ml), human epidermal growth factor (10 ng/ml) basic fibroblast growth factor (3 ng/ml) and heparin (10 µg/ml). All cells used were between passage four to nine, with media exchange after 24 hours and every 48 hours thereafter.

Scatter Enhanced Phase Contrast Microscopy

To simultaneously track cells and nanomaterials using SEPC, an Olympus ix83 inverted microscope, with phase contrast plate, was retrofitted with an oblique angle LED ring illuminator (Amscope LED-144-YK ring lamp), which attached to the microscopes brightfield condenser. Using this ring LED setup, we were able to achieve longer condenser (~3 cm) working distances as compared to traditional setups which take the place of the brightfield condenser. Typical LED powers were between 20%–50% of maximum power (Fig S6).

For phase contrast microscopy, LUCPlanFLNPh 20× and 40× objectives were used. With a numerical aperture (NA) of 0.4 and 0.65 respectively. The relatively low NA cut down on background scattering, allows for a clear DF image. For live cell imaging an Air-Therm ATX-H thermal heater coupled to a Precision Plastics stage top incubator was employed, maintaining physiological conditions (i.e. 95% humidity, 37°C internal temperatures, pH = 7.4).

Images were recorded on a Hamamatsu Orca Flash 4.0 C11440 at 16bit depth with a 0.16 µm to 0.33 µm pixel resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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and Coordination Core (ERCC) at the Center for Nanotechnology and Nanotoxicology at Harvard School of Public Health, part of the NIEHS/NHIR consortium.

Abbreviations

SEPC	Scatter Enhanced Phase Contrast
HUVECs	Human Umbilical Vascular Endothelial Cells
HASMCs	Human Aortic Smooth Muscle Cells
ENMs	Engineered Nanomaterials
DLS	Dynamic Light Scattering
DF	Darkfield Microscopy
PC	Phase Contrast Microscopy
NP	Nanoparticle
HS-DF	Hyperspectral Darkfield Imaging
LM Theory	Lorenz-Mie Theory
PDMS	polydimethylsiloxane

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Figure 1. Scatter Enhanced Phase Contrast (SEPC) allows the simultaneous visualization of cells and ENMs.

(a) SEPC light path diagram, illustrating a two-light source approach. (b) Example micrographs of HUVECs, which have been exposed to AuNPs. Micrographs obtained using darkfield (DF) (b.i), phase contrast (PC) (b.ii), and SEPC (b.iii) microscopy (scale bars, 20 μ m). (c) Line plot indicating the normalized signal intensity of each microscopy method for AuNP taken along the highlighted region. Corresponding signal-to-noise ratio measurements for SEPC and PC imaging modes using DF as a reference. (n = 13,444 particles over 11 fields of view). Indicates a marked increase in the signal-to-noise ratio.

Figure 2. Lorenz-Mie Theory can model particle DF scattering, predicting which particles can be viewed using SEPC.

(a) Calculated scattering efficiency (Q_{sca}) of example inorganic NPs. (b) Hyperspectral micrograph of AuNPs (scale bar, 5 μm)(b.i), with the corresponding single particle scattering profile (12 nm, black)(b.ii) which agrees with the LM theory predicted scattering spectrum (red)($R^2 = 0.86$). Images were contrast adjusted and smoothed for clarity. (c) Predicted NP scattering was normalized across the wavelength-dependent illumination intensity and camera recording sensitivity (d) Comparison of optical scattering intensities predicted by Lorenz-Mie theory (red) and recorded experimentally (black) for AuNPs of varying sizes, showing a good fit between theory and observation (3 s exposure time). Error bars given as the standard deviation.

Figure 3. Confirmation of NP Identity.

To confirm the highlighted objects were NPs and not cellular in origin, integrated hyperspectral darkfield (HS-DF) imaging and Raman spectroscopy were used. Au and TiO_2 NPs were used as controls for their distinct scattering (~ 520 nm) and Raman (~ 150 cm^{-1}) peaks, respectively. Representative SEPC image of HUVECs exposed to Au (a.i) and TiO_2 (b.i) NPs (scale bars, 20 μm), with the corresponding hyperspectral micrograph (a.ii) (scale bar, 5 μm) and DF regions (b.ii) (scale bar, 20 μm). (a.iii&iv.) High magnification micrograph showing the particle of interest in SEPC and HS-DF, respectively (scale bars, 1 μm). Inset images were smoothed for clarity. (a.v) Scattering spectrum of the highlighted particles (black) as compared to the average of an external control containing only AuNPs (green), indicating a good match between spectra. (b.iii) Raman spectroscopy further confirmed the ENM identity of the two particles (P1 & P2, red & black) observed under SEPC (b.i), showing a 147 cm^{-1} Raman peak, indicating the presence of TiO_2 as compared to the baseline of a cell (green), an external reference of TiO_2 NPs (red) (b.iv) and a glass substrate containing no ENMs or cells (black).

Figure 4. Tracking distinct modes of nanoparticle transport.

SEPC can be used to track different modes of aluminum oxide NP transport in cells, including intracellular transport (a), membrane assisted transport (b) and particle uptake (c). In each case, the particle of interest's transport dynamics (blue) are compared to an external background control particle (green). (i) Example SEPC micrographs showing HUVECs exposed to aluminum oxide NPs, with corresponding high magnification inset showing the position of a particle at different time points (ii). NP transport analysis, showing the instantaneous velocity of a particle (iii.), mean squared displacement (MSD) coefficient (iv), and path of travel (v), as a function of time. Inset images smoothed for clarity. (main-scale bars, 20 μ m; inset-scale bars, 5 μ m)

Figure 5. Ensemble nanoparticle dynamics using single cell composite imaging.

(a.) PDMS stamps were used to micropattern fibronectin islands (area= 2000 μm^2), yielding reproducible single cell morphologies. (b.) Example SEPC micrographs of HUVEC single cell islands internalizing TiO_2 nanoparticles. (At times: 10 min-left, 30 min-middle, 50 min-right, scale bars 25 μm). (c.) Median 2D intensity measurements of aligned and composited SEPC micrographs ($n= 3$ samples, 24 – 38 cell islands each), with the corresponding graph of normalized radial intensity, showing nanoparticle clustering in the perinuclear region as a function of time. (d.) Composite of the real time trajectories of TiO_2 NPs during uptake, showing the average velocity as a function of particle position ($n = 12$ cells, 3 samples, ~480 nanoparticles, 200 $\times$ 200 grid binning, average nucleus position highlighted in yellow, scale bar 20 μm). Perinuclear (i.), cytosol (ii.), and edge (iii.) regions indicated by white highlights. (e.) Average radial velocity (upper) and diffusivity exponent (lower) profiles showing region specific transport characteristics (errors bar given as the standard error of the mean, $n = 3$). (f.) Fluorescent micrograph of a single cell island, showing a radially aligned cytoskeleton (actin-red, fibronectin-green, nucleus-blue; scale bar=20 μm).