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**DOCTORAL THESIS STATEMENT**

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Faculty of Biomedical Engineering  
Department of Natural Sciences

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**INTERACTION OF FLUORESCENT NANODIAMONDS WITH CELLS  
INVESTIGATED BY INTERFEROMETRIC SCATTERING  
MICROSCOPY TECHNIQUES**

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# 1 Introduction

Starting from observing the stars by telescopes to imaging small structures by optical microscopes, diffraction has always been in question. There was a time when diffraction was thought to be the ultimate limiting factor to the spatial resolution of light microscopes. The first diffraction image was calculated by George Biddell Airy in 1835[1]. It was an image of a star as a point source seen by circular aperture of a telescope. The first clear mention about diffraction limitation of optical systems came much later from Émile Verdet in 1869 who based his theory on telescope viewing angle  $\omega$  and came to a conclusion that  $1/(2\omega)$  is the limitation to optical imaging. However, the most widely known work discussing the limiting role of diffraction was published by Ernst Abbe in 1873[2]. It clearly stated his theory that the optical resolution would always be limited by half-wavelength of blue light. Nevertheless, he admits that there might be way for improvement beyond those limits.

Independently on Abbe's work, Hermann von Helmholtz published his work on the same topic and matching results just one year later. In addition to Abbe's work, Helmholtz supported this theory with mathematical proofs. Another significant contribution to the topic came from Lord Rayleigh[3] who expanded the theory to various shapes of the objects and apertures. He is the author of well-known Rayleigh criterion[4] for the minimum resolvable detail given by zero distance between the first intensity minimum of one diffraction pattern and first intensity maximum of another.

The effort to surpass this diffraction limit led to development of new methods. One of the first methods with enhanced resolution but not yet transcending the diffraction limit were scanning near field optical microscopy (SNOM) using high spatial frequencies to image fine details, total internal reflection (TIRF) imaging with help of evanescent light field near cover slip to image thin layer of sample with suppressed background, confocal microscopy where a pinhole enables axial sectioning and increases lateral resolution, or methods using structured illumination[5].

Diffraction limit was finally exceeded by use of fluorescent methods. Stimulated emission depletion (STED) microscopy developed by Hell and Wichmann[6] is able to minimize a size of a fluorescent point under diffraction limit by selective depletion of fluorescence in the area while keeping the central point active. Another such technique is RESOLFT (reversibly saturable/switchable optical fluorescence transitions) microscopy first introduced by Hell and Kroug[7]. RESOLFT uses switchable fluorescence molecules similarly to STED and inhomogeneous illumination. The resolution of those techniques can go down to less than 10 nm[8], [9]. This concept of switchable optical states was later used in other techniques like

DSOM (dynamic saturation optical microscopy)[10], PALM (photoactivation localization microscopy)[11], FPALM (fluorescence photoactivation localization microscopy)[12] or STORM (stochastic optical reconstruction microscopy)[13], [14].

Optical techniques have always been popular to use in biological applications. All those enhancements gradually led to better resolution, three-dimensional (3D) imaging, multi-colour imaging and distinguishing different parts of organisms, tissues and cells[15]. It became widely used in microbiology, biotechnology, nanoscience, pharmaceutical or medical research. One of the most relevant topics these days is interaction of nanomaterials and biological systems. Nanomaterials already have a broad usage in industry and everyday products. In biomedicine, especially nanoparticles could be used for drug or contrast agent delivery into cells or as optical probes. However, this kind of biomedical applications is mostly still in the phase of research. The mechanism of interaction of nanomaterial with biological system is different from bulk material and it can show unexpected results as nano-dimension brings new properties to the material. Specific properties of nanomaterials can generate a specific response of biological system starting with interaction of nanoparticle with biomembrane and further behaviour inside the cell. The size, shape, surface characteristic and functionalisation of nanoparticle or characteristic of the environment may play a significant role in nanoparticle and cell interaction.

Knowledge of interaction between nanoparticles and cell structures is important regarding to new applications of nanomaterials, *e.g.*, imaging in living systems, biosensors, drugs delivery or toxicity analysis.[16] However, imaging in living systems has always been a challenge, especially imaging and tracking cells *in vivo*. For those purposes a suitable combination of biocompatible label and imaging techniques needs to be decided on. In this work, fluorescent nanodiamonds (fNDs) were selected as a suitable candidate for bioimaging purposes due to their biocompatible and inert nature, bright fluorescence, photostability and high refractive index. Considering those properties, a combination of interference scattering (iSCAT) microscopy, a relatively new and very promising technique, with fluorescence detection can enable unambiguous label free imaging of cell structures and long-term tracking of nanoparticles in real time.

The iSCAT characteristics such as high sensitivity, homodyne detection and unlimited observation time[17]–[19] are desired features for bioimaging or nanoparticle characterisation applications. In this work, both nanoparticle characterisation and subsequently fND interaction with living cells are successfully explored by iSCAT.

Under certain set of conditions, the iSCAT signal from a nanoparticle is proportional to the volume of the particle[20], [21] which was proven for example for metallic nanoparticles[22] or proteins.[20], [21] Here, a new approach to nanoparticle size evaluation is reported. This

approach aims to be more universal, highly sensitive and accurate and consider all possible factors for wider variety of nanoparticles and iSCAT setups. The presented method considers material properties of the scatterers and all other involved materials, illumination and optical setup properties and optical aberrations that all contribute to the iSCAT signal. Besides, it is not limited by only Rayleigh scattering and goes beyond its limit where the scattering becomes asymmetric.[23]

Because small scatterers far below the Abbe diffraction limit are used, it is not possible to obtain images of the actual shape of a nanoparticle. Thus, the method is based on determining of the vertical position of the scattering dipole of a nanoparticle placed on the cover glass calculated by rigorous theoretical model. It is demonstrated that the height of this dipole quantitatively correlates with the radius of its source.

The size measurement method is based on theoretical model and optical imaging of single nanoparticle. Similar models were presented for different purposes too, for example, to localize metallic nanoparticles,[24] to study metallic nanoparticles by photothermal microscopy[25], [26] or to study different scattering properties between dielectric and metallic nanoparticles.[27] The difference here is, that none of them adapted these theoretical models for quantitative measurement of nanoparticle size as presented in this work.[23]

After successfully characterizing fNDs with iSCAT, it was used for single particle tracking (SPT) in living cells and uncovering new findings in SPT and fND-cell interaction. Here, the combination of iSCAT with fluorescence detection came in handy when localizing even the smallest (10 nm) fNDs which opened door to unique observation of such small nanoparticle's fate inside the cell. The long-term tracking feature of iSCAT in real time enable to record short- and long-time scale events during fND-cell interaction and offered deeper insight into SPT and its data analysis. The internalization rate of bare and coated fNDs was studied. Then, the events before and after internalization were recorded showing distinct differences in fND behaviour of different sizes or functionalization.

A SPT method using fluorescence combined iSCAT for long-term observation of nanoparticles in living cells was developed including data processing and evaluation. This method is intended for wider and further use in studying living cells by iSCAT.

## 2 Aims of the thesis

The main goal of the thesis was to develop a rigorous technique for characterization of fluorescent nanodiamonds and studying their interaction with living cells.

The partial objectives to achieve this goal were:

1. *Construction of fluorescence combined iSCAT microscope*

To upgrade an iSCAT microscopy setup with fluorescence detection and set it for simultaneous detection of fND by both fluorescence and iSCAT channels.

2. *Development of theoretical model for nanoparticle characterization*

To write a theoretical model in MATLAB that will detect, localize and characterize nanoparticle size by iSCAT microscopy and to prove the functionality and accuracy of the model on measured data.

3. *Establishing a method for single particle tracking in living cells by iSCAT*

To update the iSCAT setup for long-term measurement of living cells, establish the recording conditions and suitable timescales for capturing various events of fND and cell interaction and write a MATLAB script for data processing.

4. *Studying endocytosis of fNDs and their fate in the cell*

To record and analyse different stages of fND interaction with living nonphagocytic cells with different sizes or functionalization of fNDs.

### 3 Interferometric scattering microscopy (iSCAT)

#### 3.1 State of art in iSCAT microscopy

The phenomena of interference or scattering are central principles of some already well-known microscopic techniques like differential interference contrast, phase-contrast or dark-field microscopy. The reflection interference microscopy also called reflection contrast microscopy[28]–[32] can be considered as the first precursor of iSCAT microscopy. Reflection interference microscopy was initially used for quantitative measurements on cells,[28], [33], [34] lipid bilayers[35] or phospholipid vesicles.[36] Later, coherent light source was used which significantly enhanced the contrast.[37] Interferometric scattering techniques then reached sensitivity to image particles of diameter down to 5 nm as demonstrated for gold nanoparticles[38] and been able to track single virions without any labelling.[39] The first report that used the term interferometric scattering (iSCAT) microscopy in 2009 also introduced imaging background removal method which was a significant improvement in sensitivity and precision of the method.[40]

For a long time iSCAT technique was used mainly for single particle tracking (SPT) due to its sensitivity, nanometre precision and label-free approach. First successful attempts for SPT were carried out in background-free, well-controlled, model synthetic systems like tracking virus particle[40] or gold nanoparticles (GNP)[41], [42] on supported lipid bilayers, lipids and proteins on giant unilamellar vesicles[43] or just detecting and imaging single proteins,[20], [44], [45] GNP,[46] microtubules and actin filaments in clear substrate[44], [47] and tracking metallic nanoparticles attached to motor proteins on the filaments.[47]–[49] Imaging and tracking nanoparticles in living cells is significantly more challenging due background coming from the complicated cell structures. First SPTs performed on living cells studied diffusion of intracellular vesicles[50] and membrane proteins labelled with GNPs.[49], [51] Other studies took interest in distinguishing and imaging inner cell structures like cytoplasmic organelles[52] or focal adhesions[53] since iSCAT microscopy enables label-free live cell imaging in real time with no need of staining or fixation.

In principle, the iSCAT imaging is sensitive to the difference in optical properties within the sample and any change in those properties, *e.g.*, refractive index, can be ideally detected and quantified.[54] Mass photometry[20], [21] well demonstrates the quantification of protein mass from its iSCAT contrast. Recently, the potential of nanoparticle volume quantification was expanded, and it was demonstrated that it is possible to measure volume of nanoparticles made of different materials with nanometre accuracy in a range of sizes from tens to hundreds of nanometres by iSCAT.[23]

Recently, different modalities of iSCAT microscope were introduced: Fluorescence combined iSCAT microscope that utilizes the advantages of both fluorescence and iSCAT detection,[18], [23], [53] Polarization-selective iSCAT microscopy for imaging orientation of non-spherical nanoparticles,[55] Remote focusing for 3D imaging in iSCAT microscopy[17] and resonant scattering enhanced iSCAT microscopy for resonant sensing and possible development of iSCAT spectroscopy.[56]

### 3.2 Principle of iSCAT microscopy

In iSCAT microscopy, a sample on the transparent substrate is illuminated by incident light that is then partially reflected from the glass and partially scattered by the sample. The reflected and scattered fields are collected by an objective lens and propagate along common path to the detector which minimizes the influence of external perturbations.[54] Although, the iSCAT microscope can have both reflection or transmission (also called coherent brightfield (COBRI) microscopy)[50], [57] configurations, the reflection mode is more beneficial for imaging smaller structures and objects since the incident light is only weakly reflected and does not overpower weak scattering of the subdiffraction object.[54]

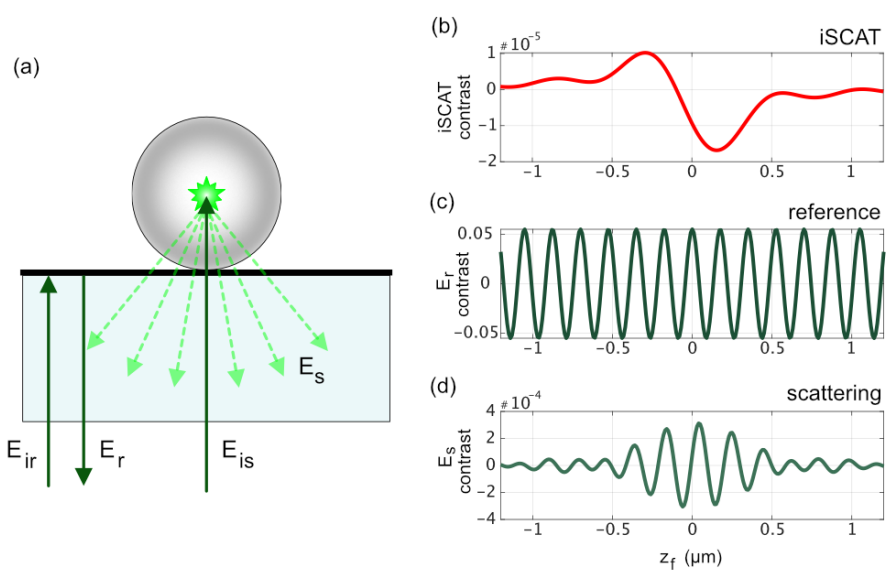


Figure 1: (a) Scheme of electric fields that generate iSCAT signal at the sample: incident beam that is scattered by the object  $E_{is}$ , back scattered field  $E_s$ , incident beam that is reflected from the cover glass  $E_{ir}$ , and reflected reference field  $E_r$ . The detected iSCAT signal (b) is then interference between reference field  $E_r$  (b) and scattered field  $E_s$  (c). (b-d) Simulation of signals: detected contrast along the focus area, i.e.  $z$  axis ( $z_f$ ).

The iSCAT signal (Figure 1) is result of the interference between the scattered field  $E_s$  by the object or structure in the sample and the reference field  $E_r$  that is the incident beam reflected from the interface between the cover glass and the sample. This can be written as:

$$I = |E_r|^2 + |E_s|^2 + 2|E_r||E_s|\cos\phi , \quad (1)$$

where  $I$  is the total intensity at a given point in the detected iSCAT image and  $\phi$  is relative phase between reflected and scattered fields.

### 3.3 Fluorescence combined iSCAT microscopy

The simplified schematics of the fluorescence-combined iSCAT microscope is pictured in Figure 2.

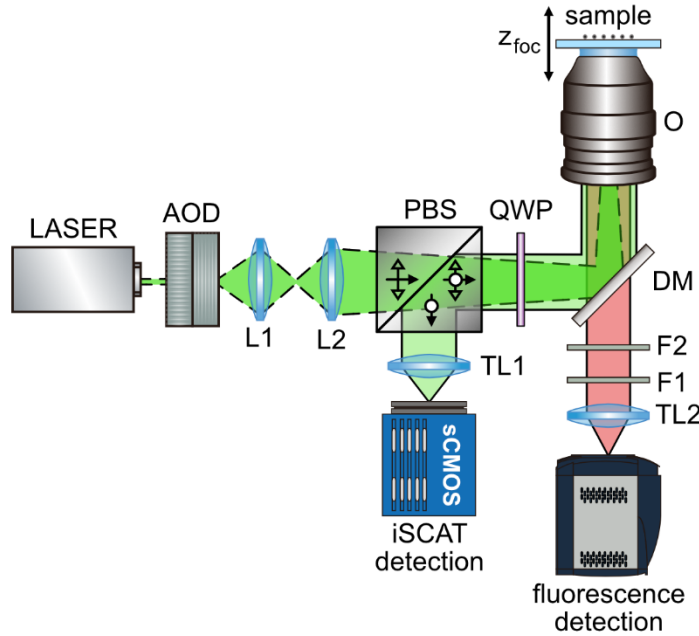


Figure 2: Fluorescence combined iSCAT microscope. The green dashed-outlined beam pathway marks the incident beam to the sample, the green solid-outlined beam pathway is then the scattered light traveling to the sCMOS detector, the red beam shows fluorescence from NPs. Optical elements in the setup are acousto-optic deflector (AOD), lenses (L1, L2, TL1, TL2), polarizing beam splitter (PBS), quarter-wave plate (QWP), objective (O), dichroic mirror (DM), notch filter (F1), and emission filter (F2).[23]

The incident 532 nm laser beam (OBIS-FP-532LX, Coherent, USA) is steered in X and Y directions by the acousto-optic deflector (AOD, DTS-XY400-532, AA Optoelectronics Ltd, France) for accurate wavefront shaping. The laser beam is then filtered and projected through the 4f telecentric lens system (L1 and L2, AC254-500-A, Thorlabs, USA), the polarizing beam splitter (PBS, CCM1-PBS25-532, Thorlabs, USA), and the quarter-wave plate (QWP, WPQ10M-532, Thorlabs, USA) onto the back-focal plane of the objective lens (O, PLAPON60XO, oil-immersion, NA = 1.42, Olympus, Japan) and sample.[23]

iSCAT detection pathway: The back-scattered light from the sample is reflected by the dichroic mirror (DM, Di03-R532-t1-25'36, Semrock, USA) and sent through a lens (TL1, same as L1) onto the sCMOS detector (pco-edge 4.2, PCO, Germany).

Fluorescence detection pathway: The emitted fluorescence from the sample is directed through a lenses (TL2, same as L1), a notch filter (F1, NF03-532E-25, Semrock, USA) and an emission filter (F2, FF01-709/167-25, Semrock, USA) to block the stray light and leakage of the excitation beam, on the EMCCD detector (iXon897, Andor, UK).

The iSCAT and fluorescence signals of the same area are detected simultaneously. The sample is held by the high precision XYZ-piezo stage (P-545.3C8S, Physik Instrumente, Germany). The back-scattered signal from a nanoparticle was recorded by moving the sample stage along its z-axis over a range of focal depth 6  $\mu\text{m}$  with the centre at the vertical position of the nanoparticle (Figure 3). The step of z-axis scanning was typically smaller or comparable to the object size, *e.g.*, for 40 nm nanoparticle the step size was 10 nm. The laser power had to be adjusted for each measurement to avoid the oversaturation of scattering or fluorescence signals. All other parameters were set to be the same for most measurements to acquire comparable data.

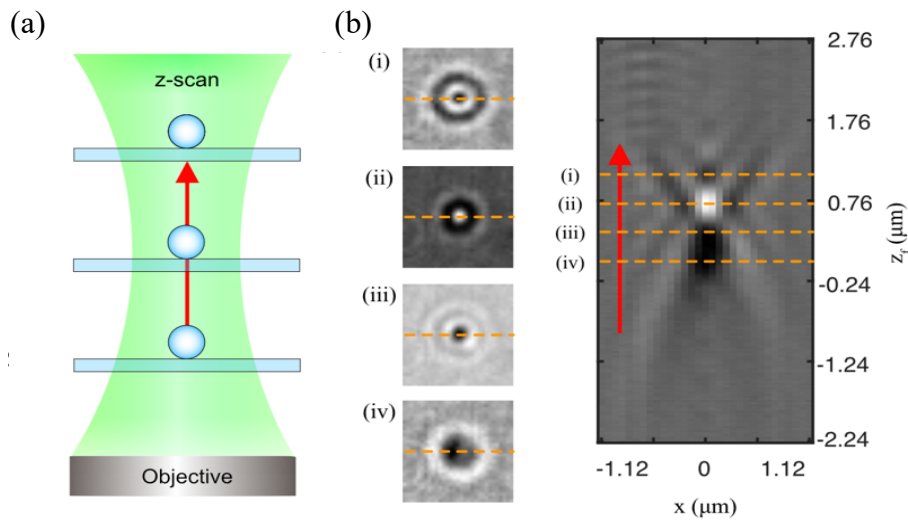


Figure 3: (a) Schematics of z-scanning in a direction of the red arrow by moving the sample stage and (b) representative example of axial variation of iSCAT image of a nanoparticle (diameter 120 nm) created by z-scanning. (i-iv) are x-y plane ROI corresponding to positions marked by orange dashed line in the x-z plane view.[23]

The experimental setup and its modifications were also described in several published works (reference [17], [23], [52], [53]).



### 3.4 Single particle tracking in iSCAT microscopy

Single particle tracking (SPT) is a powerful method to study intracellular dynamics, transport in membranes[58] or inside cells,[59] dynamics of motor proteins[60] and mapping structures[61] providing information not only about the object itself but its surrounding environment as well. The most widely used is fluorescence-based SPT that has the key advantage of easy background rejection and use of molecular-sized labels.[62] However, it has some limitations like photobleaching and blinking that result in short observation time and poor statistics in processes with fast dynamics. Additionally, saturation limit restrains the number of emitted photons and so integration times are typically longer than several milliseconds which greatly limits the spatiotemporal resolution.[46], [54]

Scattering labels can be a possible alternative to fluorescent labels. Scattering based microscopies like iSCAT does not face those limitations of fluorescence methods. Scattering signal does not bleach, blink or saturate which enables unlimited observation time and high speed and high precision tracking. On the other hand, weaker scattering of small nanoparticles and scattering background may be an obstacle when imaging especially in live cells. It is possible to increase the signal from small scatterer by increasing the laser power, but that option is limited in living cells due to phototoxicity of laser light. This limitation can be then addressed by background subtraction methods[63] and using larger labels. Although, the general rule in cell imaging is that the smaller probe the better,[46] so far it was proven that the smallest possible label to track by iSCAT was 40 nm GNP in living cell[61] and 10 nm GNP in vitro.[46]

To this date, most attention was paid to GNPs in cellular tracking by iSCAT. However, highly scattering and biocompatible fNDs have a potential to exceed the metallic nanoparticle detection limit and improve the SPT by iSCAT.

Reaching high spatiotemporal resolution is especially important to be able to observe fast and momentary events,[51], [64] e.g., endocytosis that can arise in a scale of milliseconds.[65]–[67] iSCAT microscopy enables nanometre precise tracking with sub-millisecond temporal resolution[19], [41], [68] that can reach up to microsecond resolution with accordingly fast camera.[43] This case, however, applies for detection with minimal well controlled background. Reaching such resolution in complex, highly scattering and time varying systems like living cells is more challenging.

Imaging subdiffraction objects in optical microscopy returns only finite-size PSF limited by diffraction limit which is in order of 200 nm for visible light.[54] It is possible to localize the object position more accurately by finding the centre of mass of the PSF, for example, by fitting it with Airy PSF function or two-dimensional (2D) Gaussian function. For high NA microscopes like iSCAT, Gaussian function fitting is more suitable[69] and most often used.

For shot-noise-limited detection, it is possible to reach few nanometre localization precision for number of detected photons  $N_p > 10^4$ . [70]

The particle is fitted and localized in every frame which finally reveals the whole trajectory. The trajectory can be then analysed. The parameters that are usually extracted from the trajectory data are for example distance, velocity, [71] step size, [72] diffusion coefficient  $D$ , [73] mean square displacement (MSD) [74] or the probability distribution of squared displacements (PDSD). [75]

### **3.5 Interaction of nanodiamonds with cells**

It has been observed that NDs can be successfully internalized in many types of cells. The internalization process can be spontaneous, or to trigger different types of internalization processes drugs can be used or the surface of ND can be functionalized.

The factors that affect the uptake mechanism are size, shape and surface chemistry like charge and functional groups. The preferential way of internalization of NDs smaller than 100 nm is clathrin-mediated endocytosis. [87], [98], [99] ND surface has naturally negative charge and the interaction with cell membrane is therefore most probably an electrostatic interaction. [100] Amine functionalization or transferring grafting are ways used to change the ND surface to positive charge. [100] Positively charged NDs are then most likely internalized by receptor-mediated endocytosis. [100] Other internalization pathways can be also triggered by polymer coatings such as poly-ethyleneimine (PEI) or poly-allylamine hydrochloride (PAH). [98], [99]

It was observed that inside the cells the NDs do not enter the nucleus and are mostly located at the perinuclear cytoplasm. [87] Additionally, there is difference between small and larger particles which links to their uptake mechanism. The small particles were found to be free in cytoplasm, but the larger particles were enclosed inside intracellular vesicles. [16], [98], [101]

## 4 Determination of nanoparticle size by axial profiling of interferometric scattering microscopy

These results were published in: Žambochová, K. *et al.* Axial profiling of interferometric scattering enables an accurate determination of nanoparticle size. *Optics Express* 31(6), 10101, (2023).[23]

### 4.1 Introduction

Interferometric scattering (iSCAT) microscopy has undergone significant development in recent years. It is a promising technique for imaging and tracking nanoscopic label-free objects with nanometre localization precision. The current iSCAT-based photometry technique allows quantitative estimation for the size of a nanoparticle by measuring iSCAT contrast and has been successfully applied to nano-objects smaller than the Rayleigh scattering limit. Here we provide an alternative method that overcomes such size limitations. We take into account the axial variation of iSCAT contrast and utilize a vectorial point spread function model to uncover the position of a scattering dipole and, consequently, the size of the scatterer, which is not limited to the Rayleigh limit. We found that our technique accurately measures the size of spherical dielectric nanoparticles in a purely optical and non-contact way. We also tested fluorescent nanodiamonds (fND) and obtained a reasonable estimate for the size of fND particles. Together with fluorescence measurement from fND, we observed a correlation between the fluorescent signal and the size of fND. Our results showed that the axial pattern of iSCAT contrast provides sufficient information for the size of spherical particles. Our method enables us to measure the size of nanoparticles from tens of nanometres and beyond the Rayleigh limit with nanometre precision, making a versatile all-optical nanometric technique.

### 4.2 Theoretical model development

To understand the scattering from nanoparticles and extract the information about them, we need to use an appropriate model to describe our system. A successful theoretical model should reproduce the PSF of an NP. Such a PSF model could be derived using either a vectorial or scalar approach. Scalar models derived from the diffraction theory of light use the approximation of the Fresnel-Kirchhoff integral to describe the propagation of a spherical wave through an aperture.[72] The scalar diffraction model by Gibson and Lanni (G-L) is computationally simpler and practically convenient as it directly introduces the experimental conditions as input parameters.[82], [83] It calculates the imaging aberration from the optical path difference (OPD) between the design and experimental conditions of the layers between the objective and the sample.[79]

On the contrary, the vectorial model by Richards and Wolf (R-W) is rather complicated, but it provides an accurate ray tracing method for a radiating dipole in a focused beam.[73], [74] Their vectorial approach is based on Maxwell's equations and calculates the electromagnetic field vectors,[72], [84], [85] the  $x$ ,  $y$ , and  $z$  components of which need to satisfy the corresponding wave equation.[85] The R-W model was later reformulated by Török and Varga[75] (T-V) for more general use when the electromagnetic waves are focused through a stratified medium with mismatched refractive indices. Haeberlé[82], [151] then demonstrated that such vectorial models could be used together with the G-L OPD, which provides an accurate and convenient way to model the PSF for optical microscopy. Therefore, we chose to simulate a fitting function for our experimental data with the T-V model using the G-L expression for OPD as a phase term.

#### 4.2.1 Point spread function model for iSCAT imaging

The electric field at the observation space (focal region over the detector) can be expressed in the form of the R-W integral with the additional OPD term as follows[102], [103]

$$\vec{E}(\vec{r}) = -\frac{ik}{2\pi} \int_0^{2\pi} \int_0^{\theta_{\max}} \vec{E}_{\infty}(\theta_d, \phi) e^{ik\vec{s}\cdot\vec{r}} e^{ik\Lambda_{OPD}} f(\theta_d) \sin \theta_d d\theta_d d\phi, \quad (2)$$

where  $\vec{E}_{\infty}(\theta_d, \phi)$  is the electric strength vector at the pupil of the imaging lens (far away from the focus of the lens, hence called 'far-field'),  $\vec{s}$  the unit vector of field propagation,  $\vec{r}$  the vector of the observation point,  $\Lambda_{OPD}$  the optic path difference responsible for spherical aberration,  $f(\theta_d)$  the apodization factor,  $\theta_d$  the zenith angle of the ray focused by the imaging lens, and  $\theta_{d,max}$  the semi-aperture angle of  $\theta_d$ .  $k$  is the wavenumber of the ray in the observation space, which is the same as the value in a vacuum.  $\phi$  is the azimuthal angle in the optical system and defines the azimuthal direction of a ray.  $\theta$  is the angle of a ray made with respect to the optic axis, and here it defines the polar direction of a ray in the sample space (immersion oil), which is related to  $\theta_d$  by the following equation:  $n_i \sin \theta = M n_{\text{air}} \sin \theta_d$ , where  $M$  is the magnification of the imaging system. The reference ( $E_R$ ) and scattered ( $E_S$ ) fields at the focal region of the imaging lens (over the detector) from the respective 'far-field' electric strength vectors are calculated using Eq. (2).

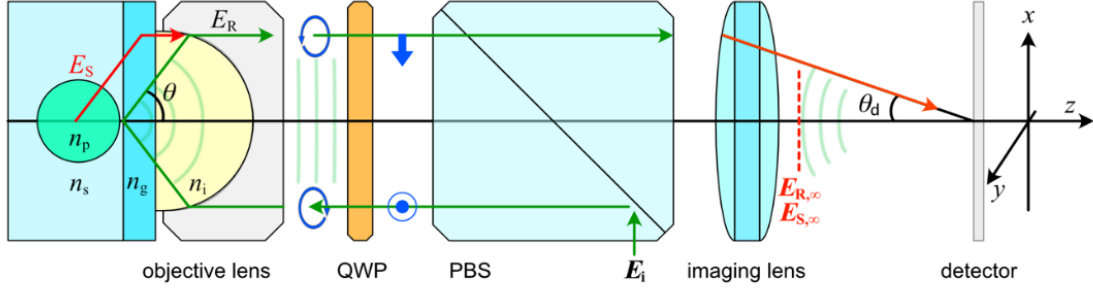


Figure 4: Illustration of the optical components of the iSCAT microscopy.[23]

To derive  $\vec{E}_\infty(\theta_d, \phi)$  for reference and scattered fields, we start with a plane wave with linear polarization ( $\vec{E}_i$ ) and trace the vectorial components of the reference and scattered electric fields by the generalized Jones matrix formalism, as illustrated in Figure 4.

The strength vector of the ‘far-field’ reference field can be expressed as follows:

$$\begin{aligned} \vec{E}_{R,\infty}(\theta_d, \phi) &= C_R \cdot i(r_p + r_s) \begin{bmatrix} -(1 - \cos \theta_d) \cos \phi \sin \phi \\ \cos^2 \phi + \sin^2 \phi \cos \theta_d \end{bmatrix} \\ &= \frac{1}{2} C_R \cdot i(r_p + r_s) \begin{bmatrix} -(1 - \cos \theta_d) \sin 2\phi \\ (1 + \cos \theta_d) + (1 - \cos \theta_d) \cos 2\phi \end{bmatrix}, \end{aligned} \quad (3)$$

where  $C_R$  is just an overall numerical factor,  $r_p$  and  $r_s$  are the Fresnel reflection coefficients at the glass-water interface and the incident field ( $\vec{E}_i$ ) is linearly polarized along the  $x$ -axis.

The strength vector of the scattered field by an NP is usually described by the Rayleigh scattering theory, which is only valid for particles smaller than the Rayleigh scattering limit. Thus, we consider the Mie theory[104] instead, for accurate modelling of the scattered field from NPs whose size is beyond the Rayleigh regime. The strength vector of the ‘far-field’ scattered field can be expressed as follows:

$$\begin{aligned} \vec{E}_{S,\infty}(\theta_d, \phi) &= C_S \cdot i(S_2 t_p + S_1 t_s) \begin{bmatrix} -(1 - \cos \theta_d) \cos \phi \sin \phi \\ \cos^2 \phi + \sin^2 \phi \cos \theta_d \end{bmatrix} \\ &= \frac{1}{2} C_S \cdot i(S_2 t_p + S_1 t_s) \begin{bmatrix} -(1 - \cos \theta_d) \sin 2\phi \\ (1 + \cos \theta_d) + (1 - \cos \theta_d) \cos 2\phi \end{bmatrix}, \end{aligned} \quad (4)$$

where  $t_p$  and  $t_s$  are Fresnel transmission coefficients at the glass and sample/water interface for p- and s-polarized light, and  $C_S$  is the overall numerical pre-factor including collection efficiency  $\eta = (1/\pi) \sin^{-1}(\min(NA/n_s, 1))$  of the objective lens along the angular extent of aperture and  $S_1$  and  $S_2$  are functions of  $\theta$  found in the scattering field components from a spherical particle obtained by the Mie theory.[104]

The iSCAT contrast depends on the phase difference between  $E_R$  and  $E_S$ , which is determined by the size and geometry of a scattering object, illumination wavelength, refractive index mismatch in the optical path, and NA.[24], [26], [27] The phase term, according to the G-L model, accounts for the aberration caused by index mismatch and finite thickness of multiple layers along the optical path.[105] The aberration can be described by considering the OPD ( $\Lambda$ ) between the real (experimental) and ideal (design) beam paths ( $\Lambda = [ABCD] - [PQRS]$ , where  $[ABCD]$  and  $[PQRS]$  are the pathlengths of real and ideal paths, respectively), as illustrated in Figure 5 and modelled in Figure 6.[105], [106]

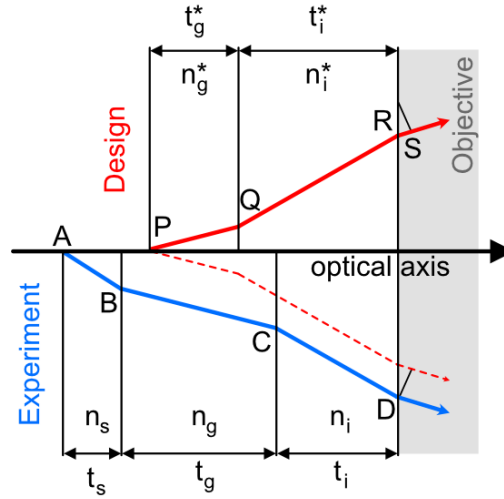


Figure 5: Depiction of the optical path of rays  $ABCD$  under experimental conditions and  $PQRS$  under design conditions.

The OPD of the reference field ( $\Lambda_{\text{OPD,Ref}}$ ) is given by those of the normally incident and reflected rays with respect to ideal rays along the same path:

$$\Lambda_{\text{OPD,Ref}} = 2(n_i t_i - n_i^* t_i^* + n_g t_g - n_g^* t_g^*). \quad (5)$$

The scattered field depends on the height of the dipole (centre position of the spherical scatterer) and thus the OPD of the field ( $\Lambda_{\text{OPD,Scat}}$ ) can be calculated using the ray geometry considered by the G-L formalism. The OPD of the scattered light is given as:

$$\Lambda_{\text{OPD,Scat}} = \Lambda(\theta, z_f, z_p, \tau) + n_s z_p + n_i t_i - n_i^* t_i^* + n_g t_g - n_g^* t_g^*. \quad (6)$$

where  $\theta$  is the angle between the ray propagation and the optical axis under which the ray is entering the objective lens;  $n_i$ ,  $n_i^*$ ,  $n_g$ ,  $n_g^*$ ,  $n_s$  are refractive indexes and  $t_i$ ,  $t_i^*$ ,  $t_g$ ,  $t_g^*$  are the thicknesses of the immersion medium (i), cover glass (g) and sample (s),  $z_p$  is the thickness of the sample if the sample lays directly on the cover glass. The asterisk \* marks the design parameter.

The reference (Eq. (3)) and scattered (Eq. (4)) fields are inserted in the R-W integral (Eq. (2)) with the aforementioned OPD terms and the integral is evaluated over  $\Phi$ . Then, the iSCAT

contrast is computed by subtracting the background from the signal and normalizing the difference with the background as:

$$I_{\text{sim}} = \frac{|\vec{E}_S|^2 + 2|\vec{E}_R||\vec{E}_S|\cos\phi}{|\vec{E}_R|^2}. \quad (7)$$

To reproduce experimental results by computation, the reference field by reflection and the scattered field from a dielectric particle was calculated as a function of the relative position of the focal plane ( $z_f$ ) and the axially stacked horizontal profiles of the reference and scattered fields (real part, phase, and amplitude) and of iSCAT ( $I_{\text{sim}}$ ) were visualized in Figure 6 (b-h).

As shown in Figure 6 (b-g), the reference and scattered fields propagate with the planar and quasi-spherical wavefronts, respectively. Moreover, the SHIP images and their axial cuts (axial variation of iSCAT signal at the centre of an NP) from the actual measurement and numerical computation look almost identical, as illustrated in Figure 6 (h) and (i). This analysis showed that it is possible to produce simulation results highly similar to experimental observations with only a specific set of parameters, indicating that the approach presented in this work is robust and reliable (Figure 6 (j)).

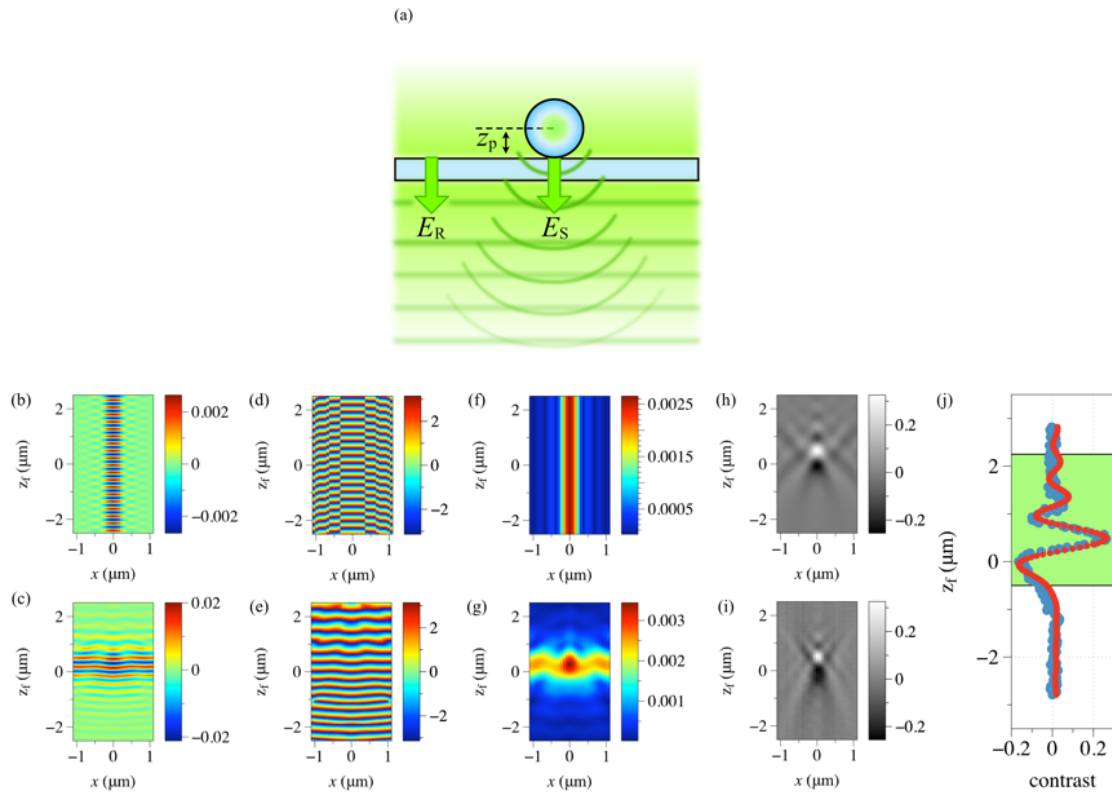


Figure 6: (a) Propagation of the scattered field ( $E_S$ ) from a nanoparticle and the reference field ( $E_R$ ) from the sample-surface interface. (b-i) Images of axially (as a function of  $z_f$ ) stacked horizontal profiles of various field quantities: (b-c) Real part of the electric field of (b) the reflected light  $E_R$  and (c) the scattered light  $E_S$ . (d-e) Phase of the electric field of (d) the reflected light  $E_R$  and (e) the scattered light  $E_S$ . (f-g) Amplitude of (f) the reflected light,  $|E_R|$  and (g) the scattered light,  $|E_S|$ . (h-i) SHIP image of iSCAT PSF (h) by simulation and (i) by experimental measurement. (j) Axial intensity profiles at the center of a nanoparticle, i.e.  $x = 0$  (experimental: blue dots; simulation: red dots). The range of  $z_f$  used to compare the two profiles is shown in green.[23]

#### 4.2.2 Strong dependence of dipole's iSCAT signal on its height

The interferometric signal between reference and scattered fields critically depends on the OPD variation, which originates from the axial location of the NP relative to the reference interface. Thus, the location of the scattering source would affect the shape of the wavefront and thus the spatial variation of the phase. It is well known that the incident field induces and drives the dipole moment in a dielectric nanoparticle. Therefore, the propagation of fields using the PSF model discussed above was simulated in order to understand how the height of the dipole, equivalently, the radius of the spherical NP affects the phase variation or the whole signal profiles. The numerical results as a function of the height of the dipole ( $z_p$ ) are displayed in Figure 7 (a-c), together with the axial profiles for three representative values of  $z_p$  (0, 50, and 100 nm) shown in Figure 7 (d-f). As shown in Figure 7 (a) and (d),  $E_R$  has no dependence on the size of a nanoparticle as expected. On the contrary, the modulation of the scattered field slightly shifts along the axial direction ( $z_f$ ) as  $z_p$  changes as shown in Figure 7 (e) (see inset). This phase shift is responsible for the axial variation of the iSCAT signal shown in Figure 7 (f) as well as in Figure 8 and Figure 9, which show that a small change in  $n_i$  influences the phase variation of  $E_S$  significantly, indicating that  $n_i$  needs to be precisely determined for accurate measurement of  $z_p$ .

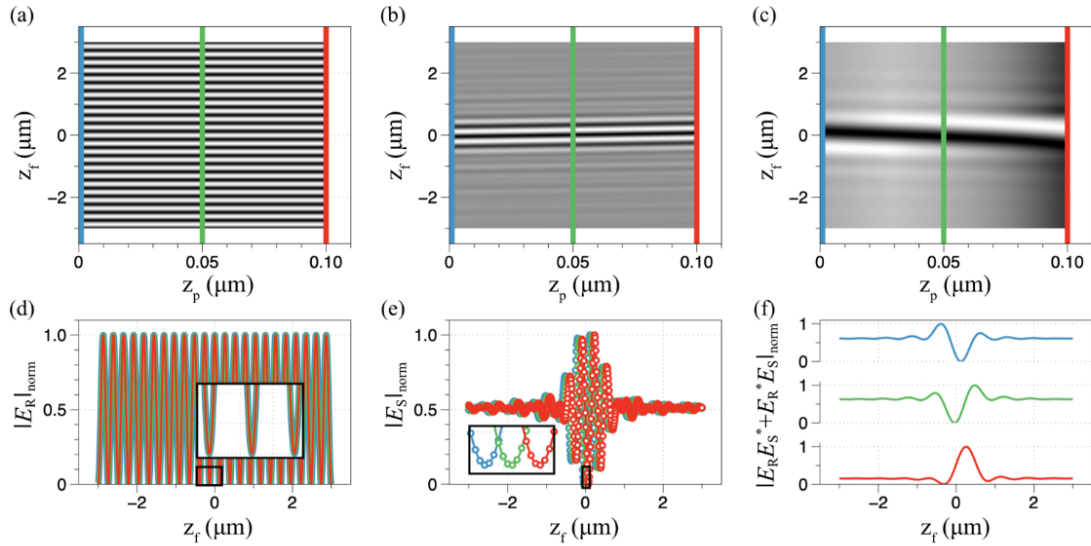


Figure 7: Stacked axial contrast profile as a function of  $z_p$ . (a)  $|E_R|$ , (b)  $|E_S|$  and (c)  $|E_R E_S^* + E_R^* E_S|$  calculated in the range of  $z_p = 0 \sim 100 \text{ nm}$  with  $n_i = 1.52$ . All calculated values are normalized and scaled to fit to  $[0,1]$  and displayed in grey scale (hence, labelled as  $|E_R|_{\text{norm}}$ ,  $|E_S|_{\text{norm}}$  and  $|E_R E_S^* + E_R^* E_S|_{\text{norm}}$  in (d-f), respectively). The blue, green and red coloured lines indicate  $z_p = 0, 50, \text{ and } 100 \text{ nm}$ . (d-f) Cross-sections of the reference field (d), the scattered field (e), and the interference term (f). Insets in (d) and (e) show the phase variation of the fields and highlight the sensitivity of the fields to different values of  $z_p$ . The axial intensity profile by the interference term exhibits more pronounced variation with  $z_p$ , accounting for sensitive detection of the size of nano-objects by iSCAT. In this figure,  $z_f$  is the computational input parameter before the offset ( $\delta z$ ) is adjusted.[23]



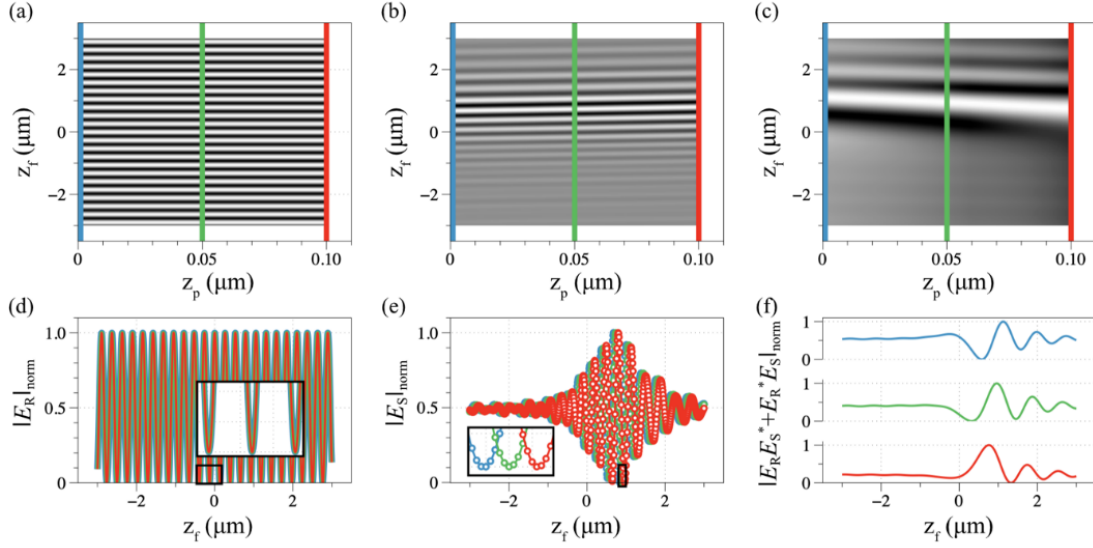


Figure 8: Simulations performed and results displayed identically to Figure 7 except for  $n_i = 1.51$ . [23]

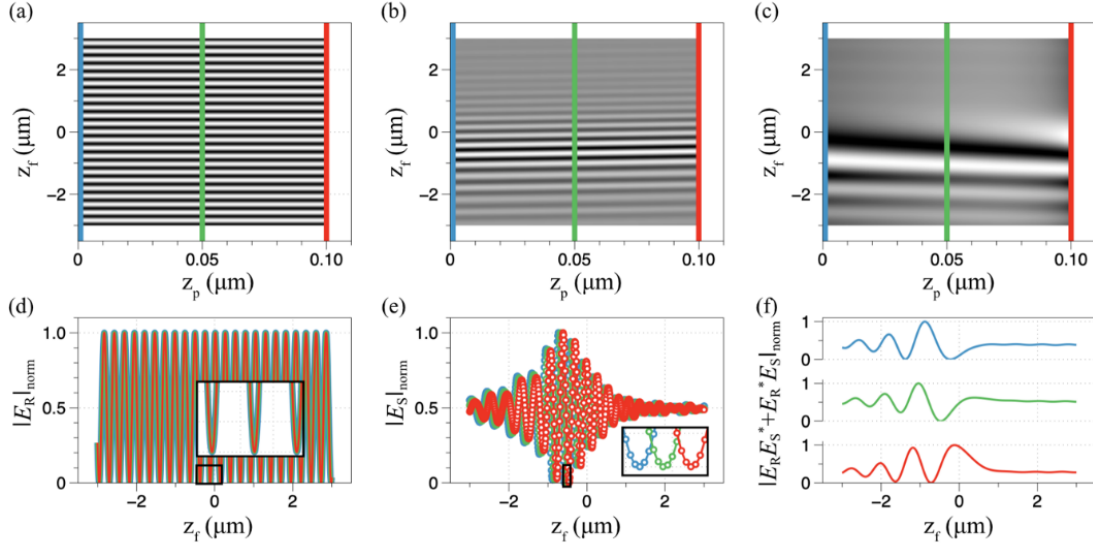


Figure 9: Simulations performed and results displayed identically to Figure 7 except for  $n_i = 1.53$ . [23]

Thus, a small change in  $z_p$  results in a notable change in the axial profile, which warrants a reliable and unambiguous analysis of experimental data. The scattering signal itself varies in amplitude, but due to its interference with the reference field, the change in the shape and amplitude of the interferometric signal becomes more pronounced. Therefore, the axial profile of the iSCAT signal significantly enhances the sensitivity to particle size. It becomes clear that our approach based on the quantitative fitting analysis of the axial pattern of the iSCAT signal can be an incisive tool for distinguishing NPs with different sizes.

#### 4.2.3 Reconstruction of axial PSF variation

The realistic model and accurate fitting of experimental data can be achieved only with fitting parameters close to reality. In the computation of PSF, the wavelength of illumination and the

NA used were 532 nm and 1.35, respectively. The refractive indexes and thicknesses used were:  $n_s = 1.33$  (for water),  $n_p = 1.59$  (for polystyrene and latex nanoparticles) or 2.4 (for nano-diamond particles),  $n_g = n_g^* = 1.52$  (for cover glass),  $n_i^* = 1.52$  (for immersion oil),  $t_g = t_g^* = 170 \mu\text{m}$  (for cover glass), and  $t_i^* = 100 \mu\text{m}$  (for immersion oil). Symbols with and without asterisk represent parameters in real (experimental) and ideal (design) conditions as used in the G-L model.[106] The  $t_i$ , ‘real’ parameter of oil thickness, is a parameter to be adjusted in the G-L model.

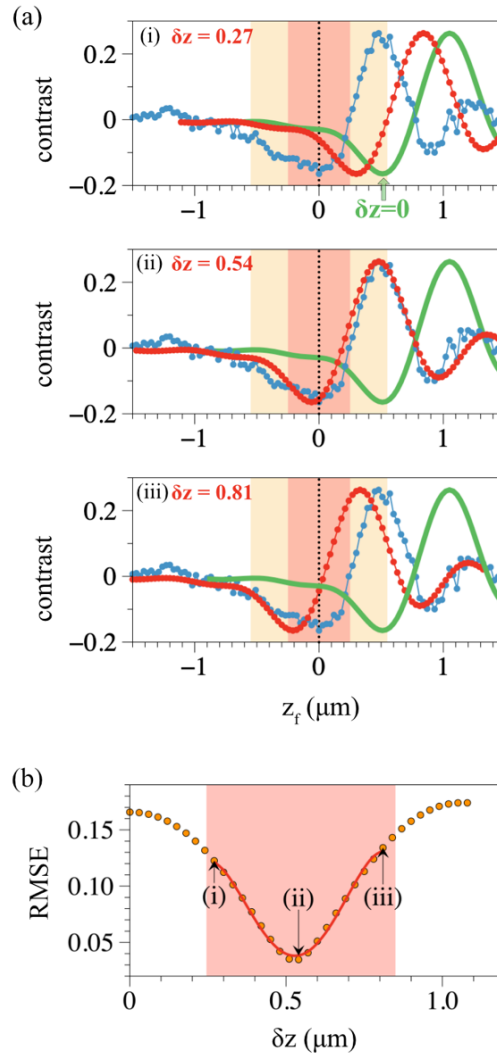


Figure 10: Root-mean-squared-error (RMSE) estimation to find the best theoretical axial intensity profile ( $I_{\text{sim}}$ ) with a given set of  $n_i$  and  $z_p$  as a function of the relative focal position ( $z$ ) that matches the experimental ( $I_{\text{exp}}$ ) profile with the highest similarity. (a) Demonstration of the profile matching procedure via translation of the simulated profile ( $I_{\text{sim}}$ ) along  $z_f$  by  $\delta z$ . The RMSE value is computed from  $I_{\text{exp}}$  (blue line with dots) and  $I_{\text{sim}}$  (red line with dots) over the shaded region with good overlap. (b) RMSE values as a function of  $\delta z$ , which is the offset applied to  $I_{\text{sim}}$ . Once the value of  $\delta z$  to yield the least RMSE is found, the offset is denoted as  $\delta z^*$ . (i)  $\delta z = 0.27 \mu\text{m}$ , (ii)  $\delta z = 0.54 \mu\text{m} = \delta z^*$ , and (iii)  $\delta z = 0.81 \mu\text{m}$  with respect to the  $\delta z = 0 \mu\text{m}$  (green solid line in (a)). We fitted the RMSE values to a 4<sup>th</sup> polynomial function to determine the representative RMSE for the given set of  $n_i$  and  $z_p$ . [23]

Parameters  $n_i$  and  $z_p$  were varied to describe the spherical aberration in the axial profile. Numerical values of these fitting parameters were determined by the root mean square error (RMSE) evaluation. First, the parameter space for  $n_i$  and  $z_p$  was defined. For each set of  $n_i$  and

$z_p$  values, the PSF (equivalently,  $I_{\text{exp}}$ ) at the focal space of the imaging lens (equivalently, on the detector surface) with various depths of the focal plane of the objective was generated from the modified R-W integral in Eq. (2). Typically,  $z$  was varied within a few  $\mu\text{m}$  from  $z = 0$ . Since only the centre value  $I_{\text{exp}}(x_0, y_0)$  was of interest, it is possible to get the axial profile of the centre of the PSF by setting  $r = 0$  in the R-W integral. This simulated PSF ( $I_{\text{sim}}$ ) is likely to be off with respect to  $I_{\text{exp}}$  due to the non-ideal experimental parameters (causing spherical aberration) and the finite size of scatterers, thus  $I_{\text{sim}}$  was shifted by  $\delta z$  to find the optimal  $I_{\text{sim}}$  with the lowest RMSE for the given  $n_i$  and  $z_p$  by compensating for a potential offset (the compensating offset giving the least RMSE is denoted as  $\delta z^*$ ). By sequentially varying  $\delta z$  (distance translated for  $I_{\text{sim}}$  with respect to  $I_{\text{exp}}$  as illustrated in Figure 10 (a)) with a fine increment within a reasonable range, one would find the least RMSE with the best  $\delta z^*$  (Figure 10 (b)) and obtain  $I_{\text{sim}}$  that best matches  $I_{\text{exp}}$  under the given set of  $n_i$  and  $z_p$ . This least RMSE is called the representative RMSE value for the given  $n_i$  and  $z_p$ .

### 4.3 Determination of nanoparticle size by axial profiling of the PSF

The RMSE estimation was carried out for axial intensity profiles generated with various values of  $n_i$  and  $z_p$  as shown in Figure 11 (a). From the minimization of RMSE, it was possible to determine the values of  $n_i$  and  $z_p$  as shown in Figure 11 (b). To validate this approach, polystyrene (PS) and latex beads were tested because they are highly uniform in size and shape (spherical), not to mention that they are homogeneous.

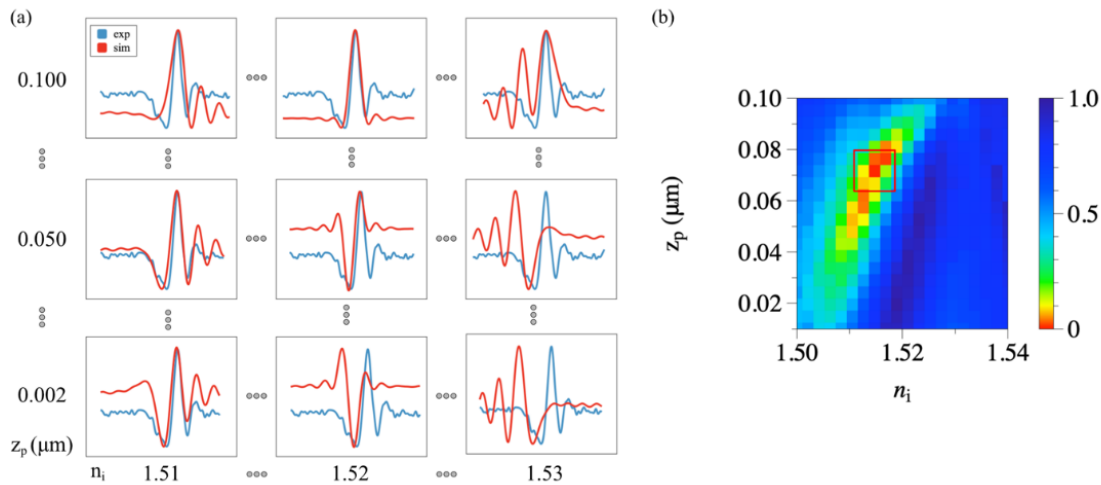


Figure 11: RMSE estimation to determine the model parameters,  $n_i$  and  $z_p$  for a 60-nm latex bead. (a) Illustration of the parameter-finding procedure by minimizing the RMSE between the measured axial contrast profile (blue line: experimental) and the computed axial contrast profile over the whole parameter space of  $n_i$  and  $z_p$  (red line: simulated). (b) Pseudo-colour maps of RMSE re-scaled to fit to  $[0,1]$  for various  $n_i$  and  $z_p$ . The minimum RMSE marked by a red box represents the best fit of the PSF modelling for the measured axial contrast profile. The  $n_i$  and  $z_p$  at the minimum RMSE are used as the parameters to generate the PSF with the highest similarity.[23]

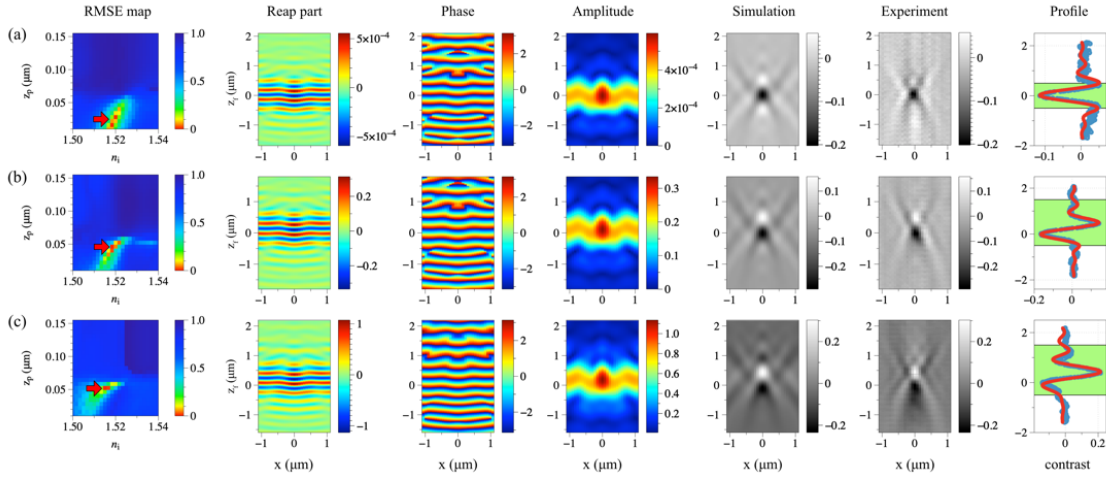


Figure 12: Representative results of PSF modelling analysis for different-sized fluorescent nanodiamonds (fND) particles from the same product number (900172, Sigma-Aldrich). This figure is presented in the same manner as Figure S7. First column: The RMSE map shows the representative RMSE values for the whole parameter space of  $n_i$  and  $z_p$ , trying to find the best fit (marked by a red arrow). Second column: Real part of the electric field of the scattered light,  $E_s$ . Third column: Phase of the electric field of the scattered light,  $E_s$ . Fourth column: Amplitude of the scattered light,  $|E_s|$ . The SHIP images, one by simulation (fifth column) and one by experiment (sixth column) with the best parameter values [(a)  $(n_i, z_p) = (1.521, 0.012 \mu\text{m})$ , (b)  $(n_i, z_p) = (1.518, 0.045 \mu\text{m})$ , (c)  $(n_i, z_p) = (1.516, 0.055 \mu\text{m})$ , with design parameters  $(n_s, n_p, n_i^*, n_g^*, t_i^*, t_g^*) = (1.33, 2.4, 1.52, 1.52, 100 \mu\text{m}, 170 \mu\text{m})$ ], exhibit clear similarity, indicating the reliability of the parameters obtained here. It is also notable that the size of fND is highly heterogeneous. The axial contrast profile in the seventh column shows the best simulation curve (red line with dots) for each experimental curve (blue line with dots). Green highlighted area defines the iSCAT features for final fitting and error calculation.[23]

Using the PSF model and adjusting the parameters carefully, it was possible to make the computational results nearly identical to the corresponding experimental data: the experimental and theoretical SHIP images are remarkably similar for different-sized beads. The size uniformity of beads was confirmed by NTA and TEM measurements. Interestingly, the results from those NPs showed that  $z_p$  determined by our method is in excellent agreement with the expected radius of those NPs. From the model fitting, the sizes of PS and latex beads were obtained, which closely match their  $R_{\text{nom}}$ 's in Figure 13 (a): (i) for 20-nm PS beads,  $z_p \sim 17 (\pm 7)$  nm; for this PS bead, the NTA method failed to give reasonable results because it is substantially smaller than the size limit for reliable measurements by NTA (dia.  $\sim 60$  nm with 642 nm excitation)[108] but TEM provided  $R \sim 23 (\pm 2.8)$  nm, consistent with the result from the PSF modelling; (ii) for 50-nm latex beads, we got  $z_p \sim 40 (\pm 1.2)$  nm while NTA and TEM gave  $R \sim 50 (\pm 18)$  nm and  $\sim 50 (\pm 3.2)$  nm, respectively, both supporting the result from PSF modelling; (iii) for 60-nm latex beads, we got  $z_p \sim 56 (\pm 10)$  nm while NTA and TEM gave  $R \sim 55 (\pm 21)$  nm and  $\sim 61 (\pm 2.5)$  nm, respectively, supporting the result from PSF modelling again; (iv) for 100-nm PS beads, we got  $z_p \sim 93 (\pm 10)$  nm while NTA gave  $R \sim 102 (\pm 20)$  nm, supporting the result from PSF modelling. As shown here, TEM is a reliable and accurate tool to measure the size of NP by direct visualization, but the limited accessibility of TEM and the low throughput and technical difficulty of the technique hampers easy and wide applications to samples in the condensed phases. This dipole-modelling-based result is consistent with our

view that the dipole of those scatterers is located near the centre of the particles and the particles are in good contact with the interface.

Next, the size of fND was estimated using the PSF model. fNDs have drawn much attention for their remarkable properties and wide applications in biological imaging and sensing.[109], [110] Theoretical SHIPs of fNDs with carefully adjusted parameters were generated, which are in good agreement with the experimental SHIPs as shown in Figure 12. The measured sizes ( $z_p$ ) of fNDs were broadly distributed, as shown in Figure 13(b), consistent with the TEM result. It, however, turned out that the size of an fND estimated by our approach was correlated with the fluorescence intensity of the fND. This issue is discussed in the next section.

Although NTA is a well-known and popular tool for size determination of NPs, it has several technical pitfalls,[111] which the method presented here could overcome. First, the size distribution measured by NTA was considerably broader than that acquired by this work's axial profile-fitting method. It is likely because the single-particle method presented here enables us to choose individual NPs and avoid clusters, significantly larger NPs, and impurities in the sample, which is intrinsically impossible with the NTA method. Second, the NTA is less straightforward in measuring the size of small NPs because instrumental settings and user inputs are critical for such NPs. While NTA could be of some use for measuring NPs of size below 60 nm, the uncertainty would be large. In contrast, the sensitivity of this method is high, so the presented approach enables accurate measurements of the size of PS beads as small as  $R=20$  nm and that of fNDs down to 15 nm in radius (Figure 13). Although several potentially useful methods have been recently developed such as iNTA that combines the advantages of NTA and iSCAT[112] and holoNTA that uses holographic imaging for larger sample volume and higher sensitivity,[113] they have not been commonly used yet.

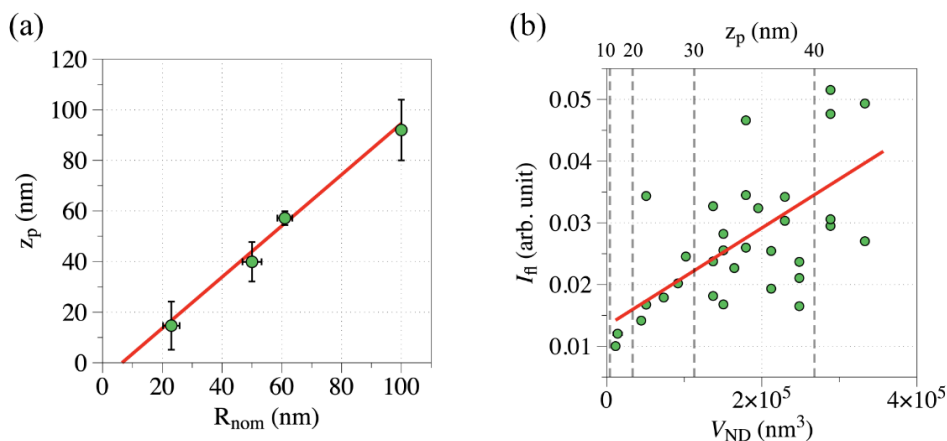


Figure 13: Representative results of particle size determination by PSF analysis. (a) Accuracy of the axial profile method to determine the size of spherical nanoparticles (PS and latex bead).  $z_p$ , radius of bead acquired by the axial profile method, is not only linearly correlated with but also nearly identical to 'Nominal radius ( $R_{nom}$ )' provided by manufacturers. Linear fit  $y = 1.0114x - 6.657$ . (b) Correlation of the fluorescence intensity ( $I_f$ ) and volume ( $V_{ND}$ ) of '40 nm' fND together with a linear fit of the data. For a better idea about the fND size, the upper x-axis shows the value of  $z_p$  determined by iSCAT. Several values of  $z_p$  are indicated by vertical dashed lines. Correlation coefficient:  $\rho(V_f, V_{ND})_{40} = 0.57$ . [23]

#### 4.4 Correlation of fND volume and fluorescence intensity

fNDs are fluorescent owing to the negatively charged NV<sup>-</sup> centres in their diamond lattice. The fluorescence intensity  $I_{fl}$  is proportional to the number of NV<sup>-</sup> centres in a given fND. From  $N_{NV} = c_{NV} \cdot V_{ND}$  ( $c_{NV}$ : concentration of NV<sup>-</sup> centres,  $V_{ND}$ : volume of fND), the fluorescence intensity should also increase linearly with the volume of fND. Regarding the correlation between them, we shall consider the following degrading factors: (i) inhomogeneous incorporation of NV<sup>-</sup> centres throughout nanodiamond lattice, (ii) the inaccurate assumption that the fNDs have a spherical shape, in fact, our fNDs appear significantly jagged as confirmed by TEM, (iii) weaker fluorescence emission by NV<sup>-</sup> centres placed near the particle surface than in the centre of the particle,[114] and (iv) batch-to-batch variation of the number of NV<sup>-</sup> centres in the fabrication process.

Figure 13 (b) shows the relationship between the volume of fND,  $V_{ND}$ , calculated from its radius,  $z_p$ , determined by iSCAT, and the fluorescence intensity  $I_{fl}$  of fND. To describe the correlation between the fluorescence intensity and fND volume, the Pearson correlation coefficient was used and the correlation  $\mathcal{R}(V_{fl}, V_{ND})_{40} = 0.57$  was obtained, which signifies only a moderate correlation. There are several particles that deviate considerably from this correlation. The main reasons for scattered points would be, as assumed above, non-uniform incorporation of NV<sup>-</sup> centres in the diamond lattice and the non-spherical shape of the nanoparticles. This implies that the size of fNDs cannot be deduced accurately from their fluorescence intensity alone. On the other hand, it was found that the distributions of fND size obtained by different methods (PSF, TEM, NTA) were well overlapped, supporting that the value of  $z_p$  (or the size of NP) acquired by the PSF model analysis is valid. From this, it can be assumed that the PSF analysis proposed here can even determine the size of NPs of non-spherical shape such as fNDs with reasonable accuracy.

#### 4.5 Conclusions

The iSCAT microscopy has evolved into a useful label-free optical technique that enables both imaging and tracking nanoscopic objects with high precision. Here, it was demonstrated that it could be used to characterize the size of NPs all-optically even beyond the Rayleigh scattering regime. A technique useful to measure the size of individual NPs by acquiring the axial variation of the iSCAT signal and fitting the theoretical PSF model to the axial profile of the iSCAT signal was developed, which provides the information on the scattering dipole position, *i.e.*, size of NPs in the present case.

The theoretical model used to fit the experimental data is a modified Török and Varga's vectorial PSF theory. This model accounts well for the factors that contribute to the iSCAT signal resulting from the interference between the scattered and reference fields. The developed method turns out to be sensitive to small changes (within a few nanometres) in the size of NPs, which surpasses other optical methods such as NTA or DLS (Dynamic Light Scattering) in size sensitivity and single-particle characterization capability, not just ensemble size distribution. It also stands out amongst other size determination techniques for its instrumental simplicity and all-optical, contactless, non-destructive, and non-contaminating approach. The results presented here demonstrate that the axial profile-fitting method is a useful approach not only because it can be used to measure the size of NP covering a broader range from  $\sim 10$  nm to several hundred nanometres, well-beyond Rayleigh scattering limit[21], [22] but also because it can be applied to NPs made of various materials. All taken together, it is anticipated that the technique presented in this work would be useful for characterizing NPs and stratified media.

## **5 Nanodiamonds for cellular imaging in iSCAT microscopy**

### **5.1 Introduction**

As a fully optical microscopic method with resolution reaching under the diffraction limit, iSCAT has a lot of potential in life cell imaging and SPT. When using iSCAT in bioimaging, there is no need for fluorescent dyes and there are almost no limitations on duration of the imaging. iSCAT offers to monitor both the probe or the object of interest and the surrounding environment at the same time. Using suitable filtering method, it is possible to obtain many information from only one data set.

In this study, the fluorescence combined iSCAT microscopy is used for SPT of fNDs in living cells to study their interaction. Particularly, it is of interest to establish a universal method to monitor fNDs of various sizes through different stages of nanoparticle-cell interaction and uncover different cell behaviour depending on nanoparticle size using that method.

The following chapters introduce SPT of fNDs in living cells from 100 nm down to 10 nm size. The differences of cell-fND behaviour between 100 nm and 10 nm fNDs are studied at different stages of their interaction. Specifically, interaction with plasma membrane, diffusion in cell cytosol and interaction with the nucleus were investigated.

Fluorescent nanodiamonds were chosen as highly suitable probe for imaging nanoparticle interaction with living cells with iSCAT. As diamond has high index of refraction, even very small particles are good scatterers and easier to detect with iSCAT technique. Especially when imaging nanoparticles in a busy and complex environment like living cell, it is important to distinguish the probe well. For complete unambiguity, fluorescence imaging was used as a reference to iSCAT to confirm the fNDs location.

### **5.2 Imaging nanodiamonds in cells**

The iSCAT image reflects diffraction patterns of every shape and surface in the cell hence the image can appear very busy at times and 3D movement of the nanoparticle can be challenging to detect. However, by using highly refractive fNDs in combination with fluorescence detection it was possible to detect fNDs from 100 nm down to 10 nm size.

Figure 14 and Figure 15 are examples of fNDs inside the U2OS cell. The left side of the images is iSCAT image and the right side is the corresponding area detected simultaneously by fluorescence channel. Figure 14 is an image with ~100 nm fND and Figure 15 image contains ~10 nm fND. It is apparent that the 100 nm fND is very clear in both iSCAT and fluorescence image and 10 nm fND not as much, yet both are detectable and traceable. The fluorescence



channel with 10 nm fND shows a lot of background noise which is caused by high illumination power needed to detect a fluorescence from such small particle with weak fluorescence. The dependence of size and iSCAT strength and fluorescence intensity was already discussed in previous chapter.

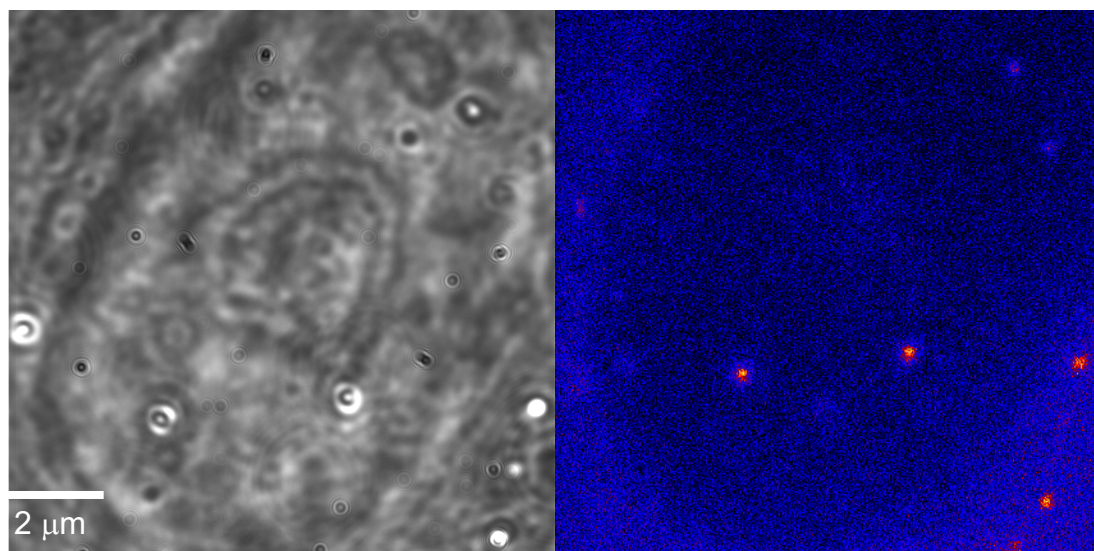


Figure 14: U2OS cell with internalized 100 nm fNDs imaged by iSCAT (left), and fluorescence detection (right), the cell nucleus area taking up most of the image is distinguished by darker fringe outline in iSCAT image and by less fluorescent area in fluorescence image.[115]

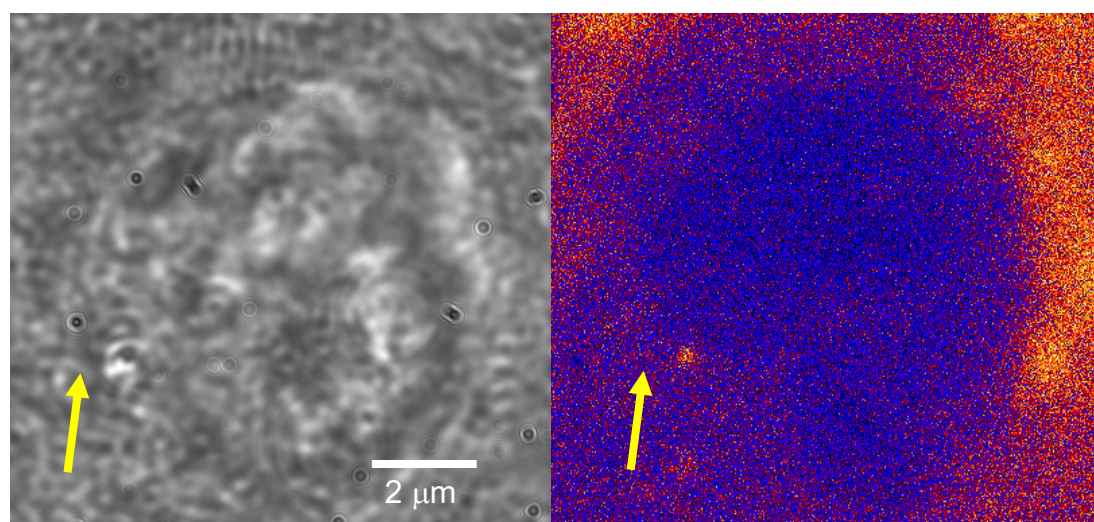


Figure 15: U2OS cell with internalized 10 nm fND (marked by yellow arrow) imaged by iSCAT (left), and fluorescence detection (right), the cell nucleus can be distinguished by darker fringe outline in iSCAT image and by less fluorescent area in fluorescence image.

The excitation wavelength was 532 nm and the detection window was defined by emission filter to be 540 – 600 nm for the fluorescence channel. In this range of wavelengths, we are able to detect the emitted signal of fND well while minimum fluorescence from the living cell is coming through. However, we are still detecting a weak fluorescence from some cell proteins at cytoplasm. The cell nucleus is free of any fluorescence hence it is clearly visible in each

image as the darkest area. On the other hand, in iSCAT image, we can distinguish more structures and their clear boundaries (Figure 16) such as nucleus, nucleolus, endoplasmic reticulum, cytoskeleton[53] or vesicles. Some studies also aim to image Golgi apparatus or mitochondria.[52] Especially in the case of imaging  $\sim 10$  nm fNDs, the fluorescence reference image comes in handy when distinguishing the fND from natural cell vesicles since their iSCAT image can look almost identical.

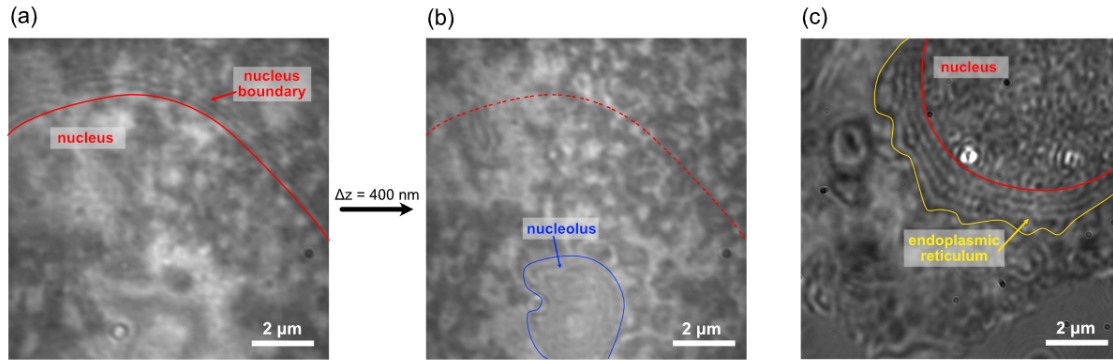


Figure 16: Cell structures visible by iSCAT: (a) upper surface of U2OS cell with visible fringe bordering the nucleus, (b) internal structure of the same cell with focus at nucleolus, (c) internal structure of another U2OS cell with endoplasmic reticulum surrounding nucleus. The focus difference between (a) and (b) is 400 nm. The bright scattering spots in (c) are internalized 10 nm fNDs.

Figure 16 is example of U2OS cell with some visible structures. Figure 16 (a) and (b) are images of the same cell captured at different heights with difference 400 nm. Image (a) is captured at the upper surface of the cell where the nucleus boundary is recognized by diffraction fringe signaling steeper slope of the cell surface. When the focus is moved down closer to the cover glass, the internal structures appear more clearly a we can observe nucleolus in the image (b). In Figure 16 (a), the nucleus is clearly visible at the top right quarter of the image. When focused inside the cell, the nucleus always appears as the part of the cell with the densest structure in iSCAT. Around the nucleus border, endoplasmic reticulum is well visible in this image. The border of the cell can be seen at the bottom right of the image. The smooth areas are the surface of the culture dish.

### 5.3 Development of nanoparticle tracking software for iSCAT imaging

There is some tracking software freely accessible, for example tracking add-in for ImageJ, however, these are meant for localizing fluorescence or absorption imaging data and are not suitable for the complex iSCAT images presented in this work. Therefore, an appropriate tracking software had to be developed specifically for our type of data. The tracking software was made using MATLAB graphical user interface (GUI). The main features of the software are particle detection, automatic particle tracking, image processing and data processing.

The tracking interface is shown in Figure 18. This software enables data postprocessing after the acquisition of the data. First, the source video, in this case iSCAT recording, is opened which is visualized in the top left corner. By clicking the particle that we want to track, we select the position of ROI. The size of ROI can be adjusted to the size of the nanoparticle PSF size. The particle PSF is then fitted by gaussian function and it is centered in the ROI for each frame. The image of the particle PSF and gaussian fitting can be seen below the main video. Additional parameters of the gaussian fit of ROI, i.e. number of particles in ROI, how many frames are accumulated for the fitting and the tolerable RMSE threshold, can be adjusted. Next, the image is normalized and static background is removed by subtracting consecutive images. Figure 17 shows the iSCAT image corresponding to the frame in Figure 18 before the normalization and background subtraction. After such filtering we get much clearer image of the moving particle. In case the particle stops moving or escapes from the frame by rapid moving in z-axis direction, we are able to maintain the trajectory by gap-filling with the simultaneously recorded fluorescence detection which has broader detection depth than iSCAT. Although the software was developed for iSCAT data processing, any other type of imaging, like fluorescence in this case, can be analysed by it as well.

While this software is able to track the nanoparticle only in x-y plane, the z-axis data can be additionally extracted either from the particles PSF image[116] or it can be recorded by remote focusing[17] while maintaining the particle in focus in real time. Nevertheless, the data presented in this work are only 2D x-y trajectories whilst 3D tracking is still in development.

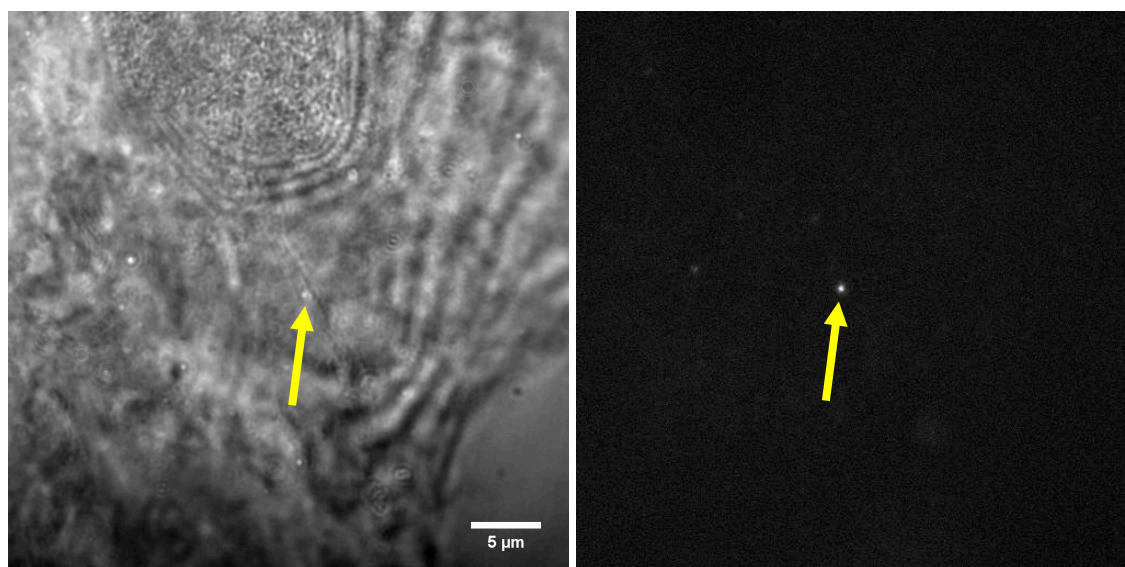


Figure 17: The iSCAT source recording used for the analysis example in Figure 18 before filtering out the background (left) and the complementary fluorescence recording (right). Yellow arrow marks the position of the tracked fND.



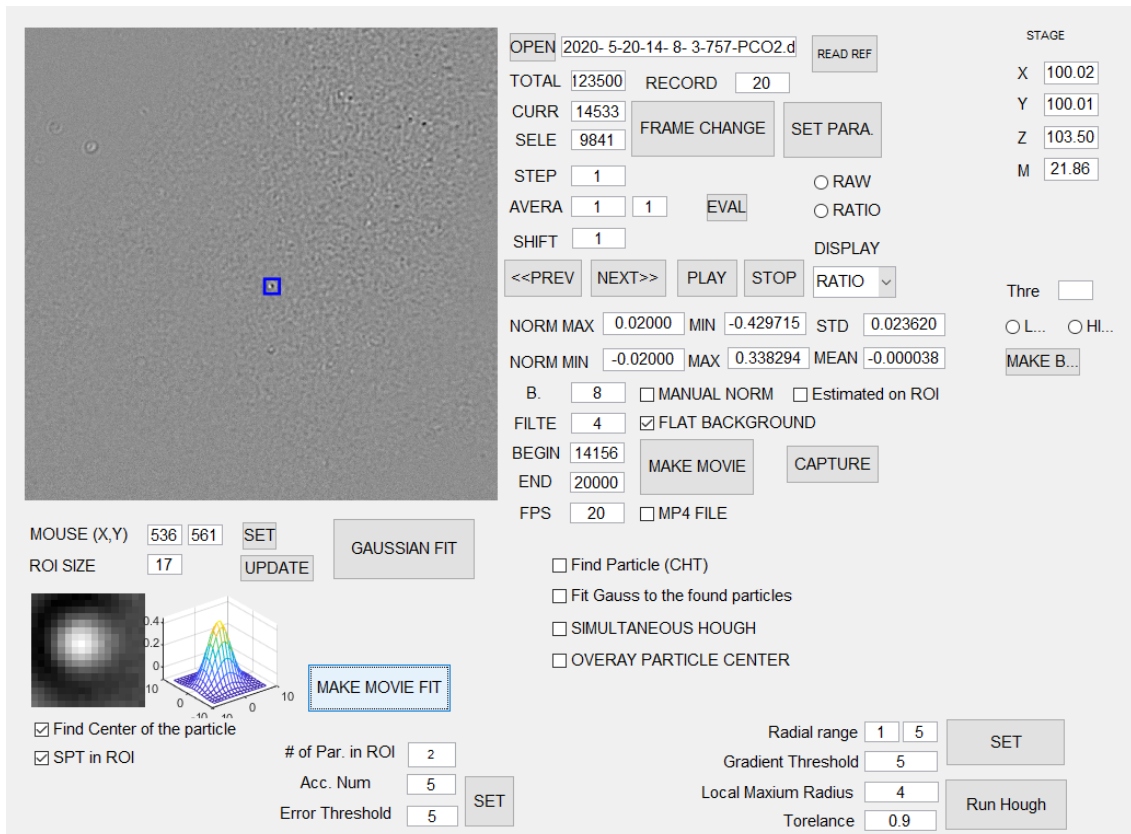


Figure 18: Particle tracking software made in MATLAB graphical user interface (GUI). The GUI involves display of the source recording, controls for preview of the recording, the particle detection window and fitting, filtering options and analysis controls.

## 5.4 Diffusion of fNDs in cytosol

Different sizes of nanoparticles should exhibit different speed and types of motion. Here, two sizes of fNDs were tested, the smallest possible to detect with iSCAT, 10 nm fNDs, and 100 nm fNDs which are larger enough and should be well received by cell uptake mechanism.

The trajectory of fND was recorded first and analysed afterwards. The analysis involved plotting an MSD curve, fitting the short time-lag data (the first linear section of the MSD curve) and the long time-lag data (second linear section of the MDS curve). The rest of the data was excluded from the analysis to minimize the error because less data is collected for long time-lags and the uncertainty of MSD analysis is therefore higher, as explained in the theoretical section 3.4.

### 5.4.1 10 nm fND diffusion

Twenty 10 nm fND particles were analysed in total. The types of motion detected can be sorted into four different categories of motion: (i) normal diffusion, (ii) anomalous diffusion, (iii) confined diffusion and (iv) directed motion. Additionally, each type of diffusion seemed to be typical for different parts of the cell. In general, the closer to the nucleus the more confined the

motion seemed to be. One of the reasons can be the fact that the cell is more occupied by organelles and intrinsic structures towards the nucleus opposed to the outskirts areas.

The diffusion of 10 nm fND near the nucleus is typically closely confined. Figure 19 demonstrates two cases of such motion. Pictured fND #1 diffuses right along the border of the nucleus in anomalous manner. Both from the trajectory and MSD curve it is recognizable that the particle diffuses in a confined prolonged area, but it is not completely trapped. Such particle can be for example confined in between organelles surrounding the nucleus, *e.g.*, Golgi apparatus. While fND #2 is enclosed in much smaller area. Such particles exhibiting minimal motion and slow diffusion are typically found right on the surface of the nucleus. While it is believed that particle of this size cannot enter the nucleus itself, it can enter the perinuclear area. The shape of the MSD curve clearly shows a confinement that can be recognized by the second segment having lower slope than the first segment.

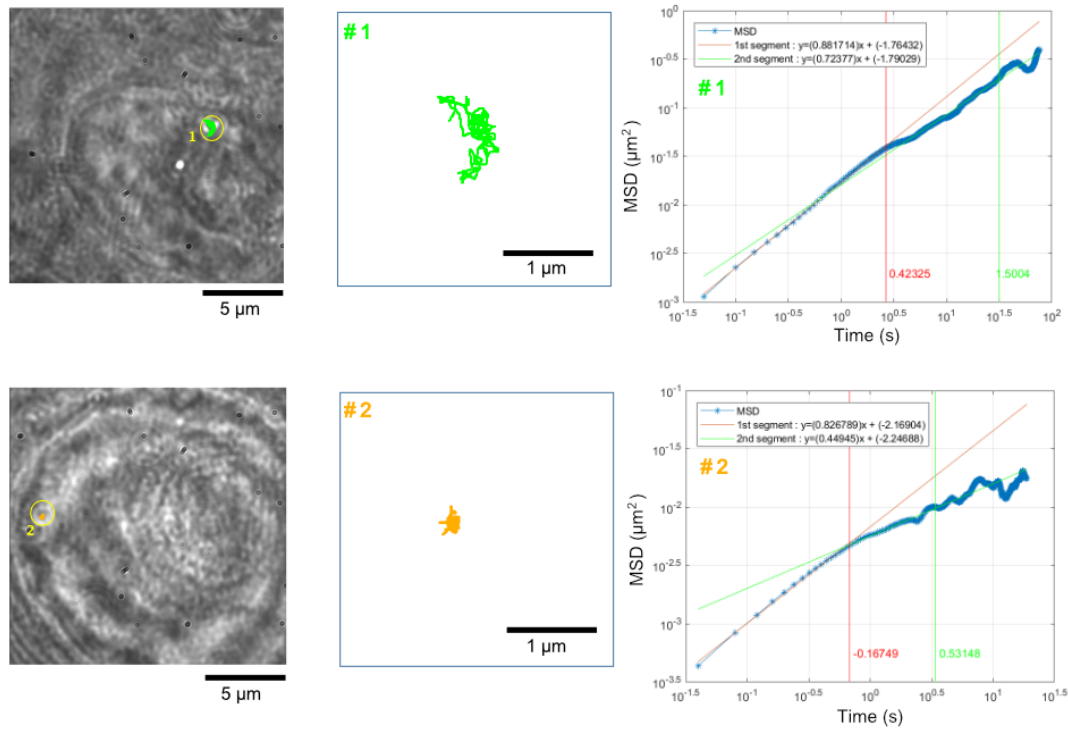


Figure 19: 10 nm fND diffusion in the area surrounding the nucleus and corresponding MSD analysis. (a) image of the position of the fND #1 and #2 inside the cell. (b) 2D Trajectories of the fNDs #1 and #2. (c) MSD curves (blue) corresponding to the trajectories #1 and #2. The red fitting line fits the short time-lag (1<sup>st</sup> segment) data and the green fitting line fits the long time-lag (2<sup>nd</sup> segment) data. Each of the time-lag section is designated by the vertical line of the corresponding colour. Legend: fND #1 1<sup>st</sup> segment:  $y = 0.88x - 1.76$ , 2<sup>nd</sup> segment:  $y = 0.72x - 1.79$ . fND #2 1<sup>st</sup> segment:  $y = 0.83x - 2.17$ , 2<sup>nd</sup> segment:  $y = 0.45x - 2.25$ .

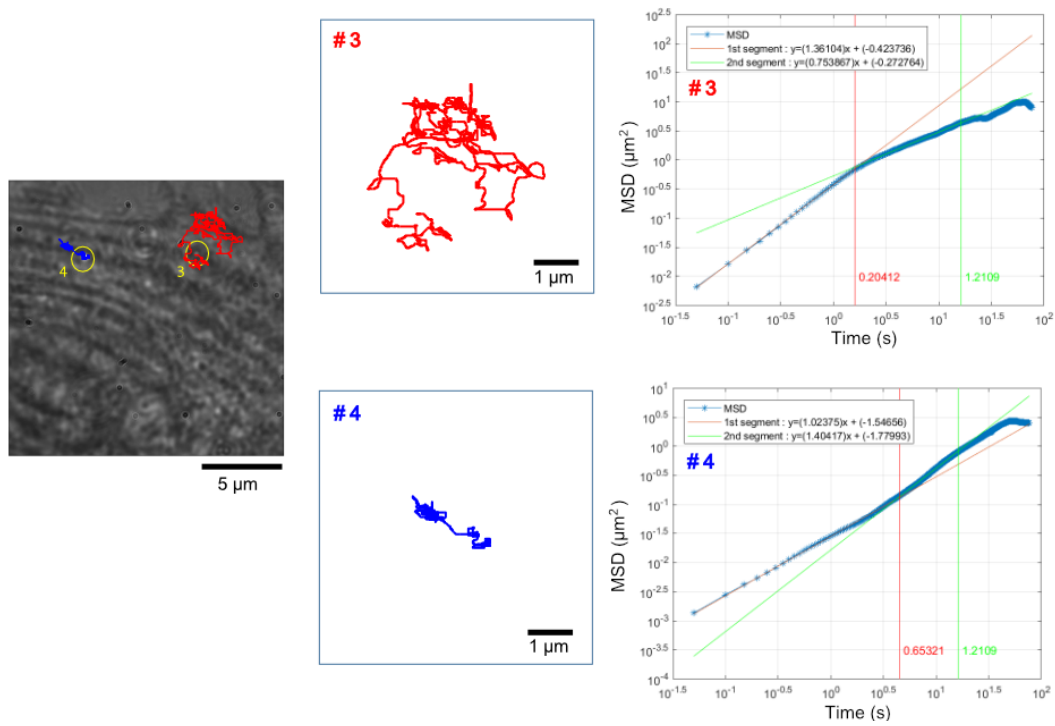


Figure 20: 10 nm fND diffusion in cytosol and corresponding MSD analysis. (a) image of the position of the fND #3 and #4 inside the cell. (b) Trajectories of the fNDs #3 and #4. (c) MSD curves (blue) corresponding to the trajectories #3 and #4. The red fitting line fits the short time-lag (1<sup>st</sup> segment) data and the green fitting line fits the long time-lag (2<sup>nd</sup> segment) data. Each of the time-lag section is designated by the vertical line of the corresponding colour. Legend: fND #3 1<sup>st</sup> segment:  $y = 1.36x - 0.42$ , 2<sup>nd</sup> segment:  $y = 0.75x - 0.27$ . fND #4 1<sup>st</sup> segment:  $y = 1.02x - 1.55$ , 2<sup>nd</sup> segment:  $y = 1.40x - 1.78$ .

The particles fND #3 and #4 in the Figure 20 represent typical motions of 10 nm fNDs in the cytosol. fND #3 is diffusing freely in the cytoplasm right at the outskirts of the cell. The range of the motion of #3 is the largest and its diffusion is the fastest of all pictured examples. fND #3 is the closest to the Brownian motion while the MSD curve still indicates that the motion was, in fact, confined in the long-time scale. Last but not least, fND #4 is an example of the consecutive confined motion areas with fast somewhat directional transitions in between them, which is called corralled diffusion. A particle can undergo this kind of diffusion either when it diffuses in between cellular organelles by itself or when it is trapped in a vesicle which can be transported by active transport mechanism of the cell. In this particular case, the MSD curve shape suggests the active transport involvement since the second segment of the curve has higher slope than the first meaning fast diffusion in long time scale.

#### 5.4.2 100 nm fND diffusion

Fifty 100 nm fND particles were analysed in total. The detected types of motion were confined diffusion, anomalous diffusion and directed motion. In comparison to 10 nm fNDs, no free diffusion was observed. The most common type of motion for 100 nm fNDs was the corralled diffusion. It can be detected both in cytosol and near the nucleus.

In the Figure 21 all three pictured fNDs are in the vicinity of the nucleus. The fNDs #5 and #6 both exhibit the corralled diffusion. Meanwhile, fND #7 undergoes confined diffusion very similar to 10 nm fND #1 in scale, placement and shape of the MSD curve. It is important to note, that the timescale for these three particles is the same. All three MSD curves suggest the anomalous diffusion, which is distinguished in the long-time scale, for #5 and #7 it becomes confined diffusion, for #6 it is confined transitioning to directed diffusion.

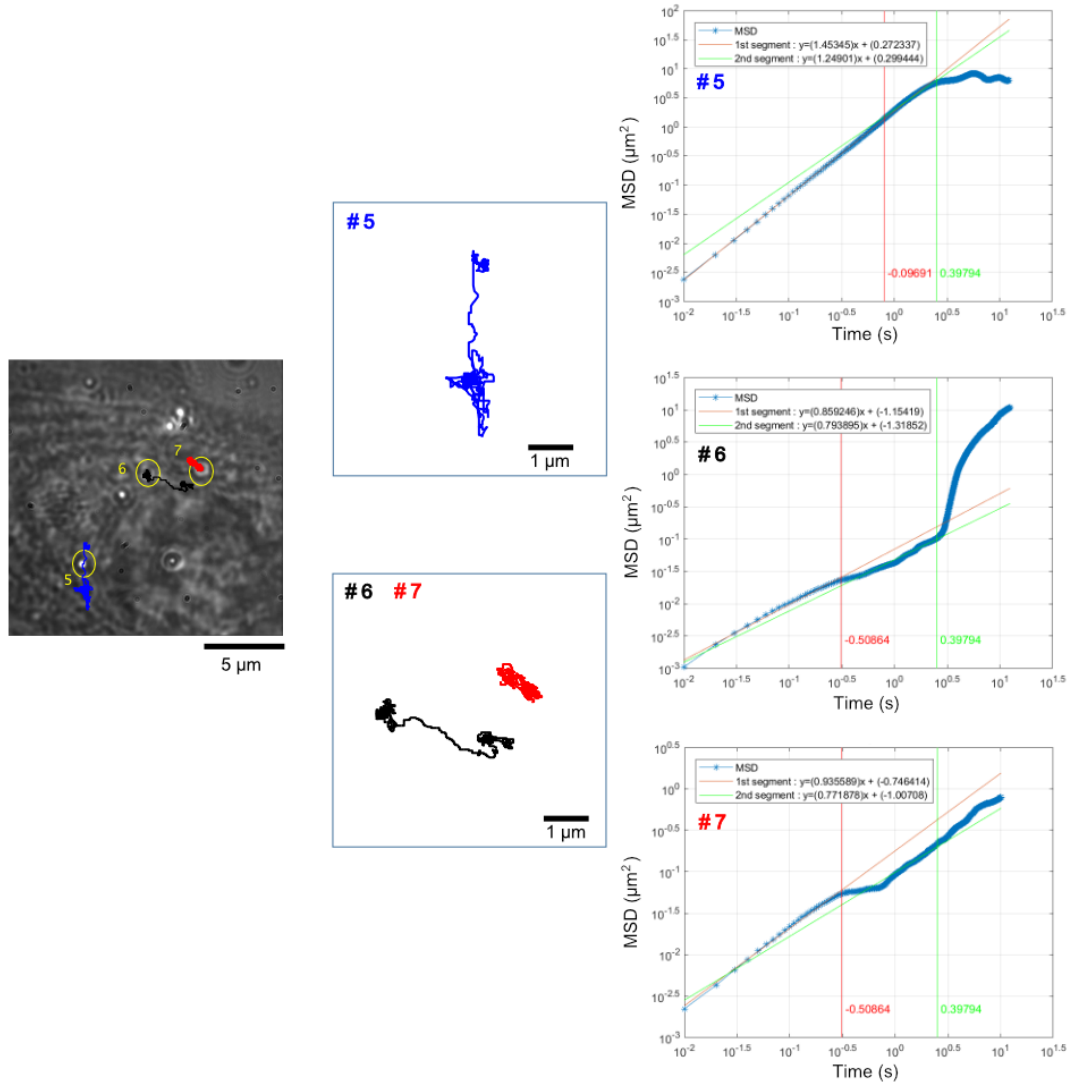


Figure 21: 100 nm fND diffusion near the nucleus and corresponding MSD analysis. (a) image of the position of the fND #5, #6 and #7 inside the cell. (b) Trajectories of the corresponding fNDs. (c) MSD curves (blue) corresponding to the trajectories. The red fitting line fits the short time-lag (1<sup>st</sup> segment) data and the green fitting line fits the long time-lag (2<sup>nd</sup> segment) data. Each of the time-lag section is designated by the vertical line of the corresponding colour. Legend: fND #5 1<sup>st</sup> segment:  $y = 1.45x + 0.27$ , 2<sup>nd</sup> segment:  $y = 1.25x + 0.30$ , fND #6 1<sup>st</sup> segment:  $y = 0.86x - 1.15$ , 2<sup>nd</sup> segment:  $y = 0.79x - 1.32$ , fND #7 1<sup>st</sup> segment:  $y = 0.94x - 0.75$ , 2<sup>nd</sup> segment:  $y = 0.77x - 1.01$ .

The fND #8 in the Figure 22 is diffusing inside the cytosol and it is exhibiting fast directional motion with very few confinements along the way. Both the trajectory and the MSD curve show typical shapes for the directed motion. #9 is an example of the corralled diffusion with directional motion in the cytosol. fND #10 is located on top of the nucleus and same as the

10 nm fND #2 it exhibits very confined motion which brings us to believe that even 100 nm fND can be trapped in the perinuclear space.

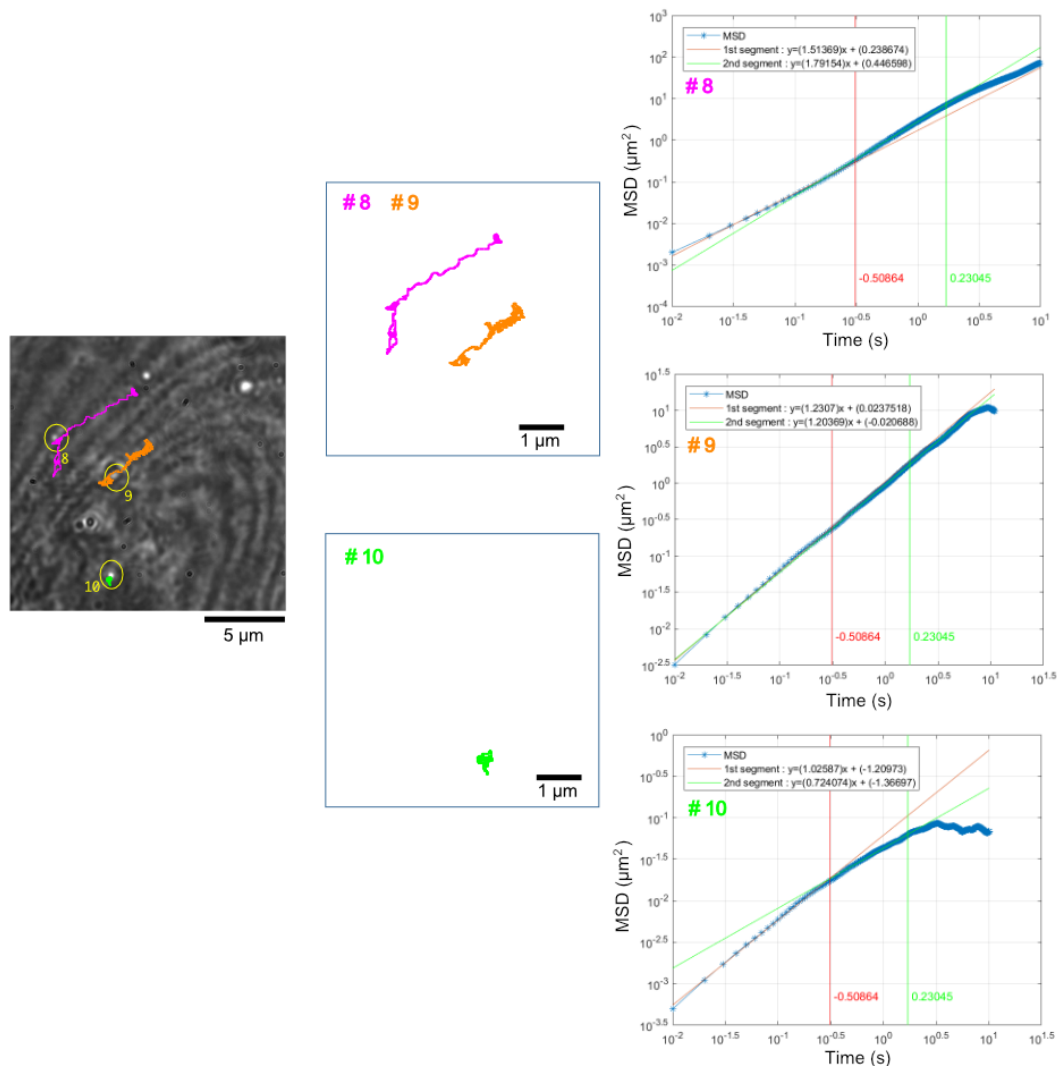


Figure 22: 100 nm fND diffusion and corresponding MSD analysis. (a) image of the position of the fND #8, #9 and #10 inside the cell. (b) Trajectories of the corresponding fNDs. (c) MSD curves (blue) corresponding to the trajectories. The red fitting line fits the short time-lag (1<sup>st</sup> segment) data and the green fitting line fits the long time-lag (2<sup>nd</sup> segment) data. Each of the time-lag section is designated by the vertical line of the corresponding colour. Legend: fND #8 1<sup>st</sup> segment:  $y = 1.51x + 0.23$ , 2<sup>nd</sup> segment:  $y = 1.79x + 0.45$ , fND #9 1<sup>st</sup> segment:  $y = 1.23x + 0.02$ , 2<sup>nd</sup> segment:  $y = 1.20x - 0.02$ , fND #10 1<sup>st</sup> segment:  $y = 1.03x - 1.21$ , 2<sup>nd</sup> segment:  $y = 0.72x - 1.37$ .

Overall, it seems that 100 fNDs are undergoing corralled diffusion often combined with some directed motion. This suggests that 100 nm fNDs are enclosed in intracellular vesicles and those vesicles can be part of the active transport of the cell. This behaviour is different from the 10 nm fNDs where we observed confined diffusion in a very tight spaces or Brownian motion in a larger space with obstructions which leads us to believe that 10 nm fNDs are not trapped in a vesicle but more likely diffuse by themselves.



### 5.4.3 Diffusion coefficient of fND in the U2OS cell

The diffusion coefficient of fND examples above is in the Table 1. The diffusion coefficients were calculated from the first segment of the MSD curves for short time-lags using the formula for anomalous diffusion (Eq. (32)).

Table 1: Typical values of 2D diffusion coefficient of 10 and 100 nm large fNDs diffusing in the cell cytosol and nucleus. Particle number corresponds to the numbering in Figure 19 - Figure 22.

| Particle number | Size | Location | Diffusion type | 2D diffusion coefficient (nm <sup>2</sup> /s) |
|-----------------|------|----------|----------------|---|
| 1               | 10   | Nucleus  | Anomalous      | 4.05  |
| 2               | 10   | Nucleus  | Confined       | 1.69  |
| 3               | 10   | Cytosol  | Free           | 9.42  |
| 4               | 10   | Cytosol  | Corralled      | 7.10  |
| 5               | 100  | Cytosol  | Corralled      | 71.1  |
| 6               | 100  | Cytosol  | Corralled      | 67.4  |
| 7               | 100  | Cytosol  | Confined       | 24.0  |
| 8               | 100  | Cytosol  | Directional    | 433   |
| 9               | 100  | Cytosol  | Directional    | 264   |
| 10              | 100  | Nucleus  | Confined       | 15.4  |
| 11              | 100  | Nucleus  | Confined       | 1.99  |

The Table 1 represents typical values measured across all seventy analysed fNDs. Particle #11 is not displayed amongst the visual examples above, however, it was included in the table as an important representation of 100 nm fND sample.

The 2D diffusion coefficients show that

- the diffusion in the proximity of the nucleus is significantly slower,
- the 10 nm fND diffuses several times slower in the cell than 100 nm fND,
- the directional motion where the active transport is involved can be ten times faster than the other passive diffusion types.

A few principles can explain these behaviours. A particle diffuses slower in an environment with higher mass density which is seen in the case of the particles near the nucleus. Here we have two cases of the diffusion in the nucleus proximity. One, tightly confined particles (#2, #10 and #11) right at the border of the nucleus, which is believed to be a fND trapped in the perinuclear space. There, besides the small space for the diffusion, the density of the perinuclear

cytoplasm is about two times higher[117] than the cytoplasm which makes the diffusion also slower. Second, anomalous diffusion which is confined with wider range of movement at the nucleus proximity (#1). While #1 has higher diffusion coefficient than tightly confined #2 it is still twice slower than fNDs diffusing in the free cytoplasm showing that even with less confinement, the particle is slowed down in the perinuclear environment.

The fact that smaller fNDs had slower diffusion than large fNDs can be, in general, caused by small particles being easily trapped in crevasses and small structures that do not cause any obstruction to the particles that are larger than the possible entrapment. This also results in smoother trajectories for the 100 nm fNDs. Other reason for the fasted diffusion of 100 nm fND is its fate in the cell. As already established, the 100 nm fND can be encapsulated in the vesicle after clathrin or calveolin mediated endocytosis, thus undergoing smoother transport in the cell including active transport.

## **5.5 Interaction of fNDs with plasma membrane**

The interaction with a cell membrane was recorded two hours after introducing the fNDs to the cell culture medium. As internalization rate experiment showed, no fNDs should be internalized yet. At this point, it was possible to observe free fNDs in the cell culture medium, fNDs attaching and detaching from the cell membrane and fNDs diffusing along the membrane surface which consequently leads to the internalization process. The particles attached to the membrane were the observation target here. The aim was to monitor the events before the internalization and general particle behaviour on top of the membrane for comparison with its behaviour inside the cell.

To capture as many events as possible, the measurement was carried out for as long as possible. The main restriction was the cell vitality. To keep the cell alive and vital for as long as possible, it was placed in small incubation cell which was anchored to the microscope's sample stage. The cell was enclosed and kept atmosphere with 5 % CO<sub>2</sub> and temperature 37 °C. Another condition was to keep the laser power at the lowest level possible. The laser power sufficient to detect the fND was 0.4 – 1.3 mW at the sample. In this setting, the recording time possible was two hours before the cell death. However, changes in cell vitality were usually detectable already after one hour. Therefore, the results presented here are only acquisitions from the first hour of the recordings. The sample chosen for this experiment were 100 nm fNDs due to their easy detectability in iSCAT and high internalization rate.

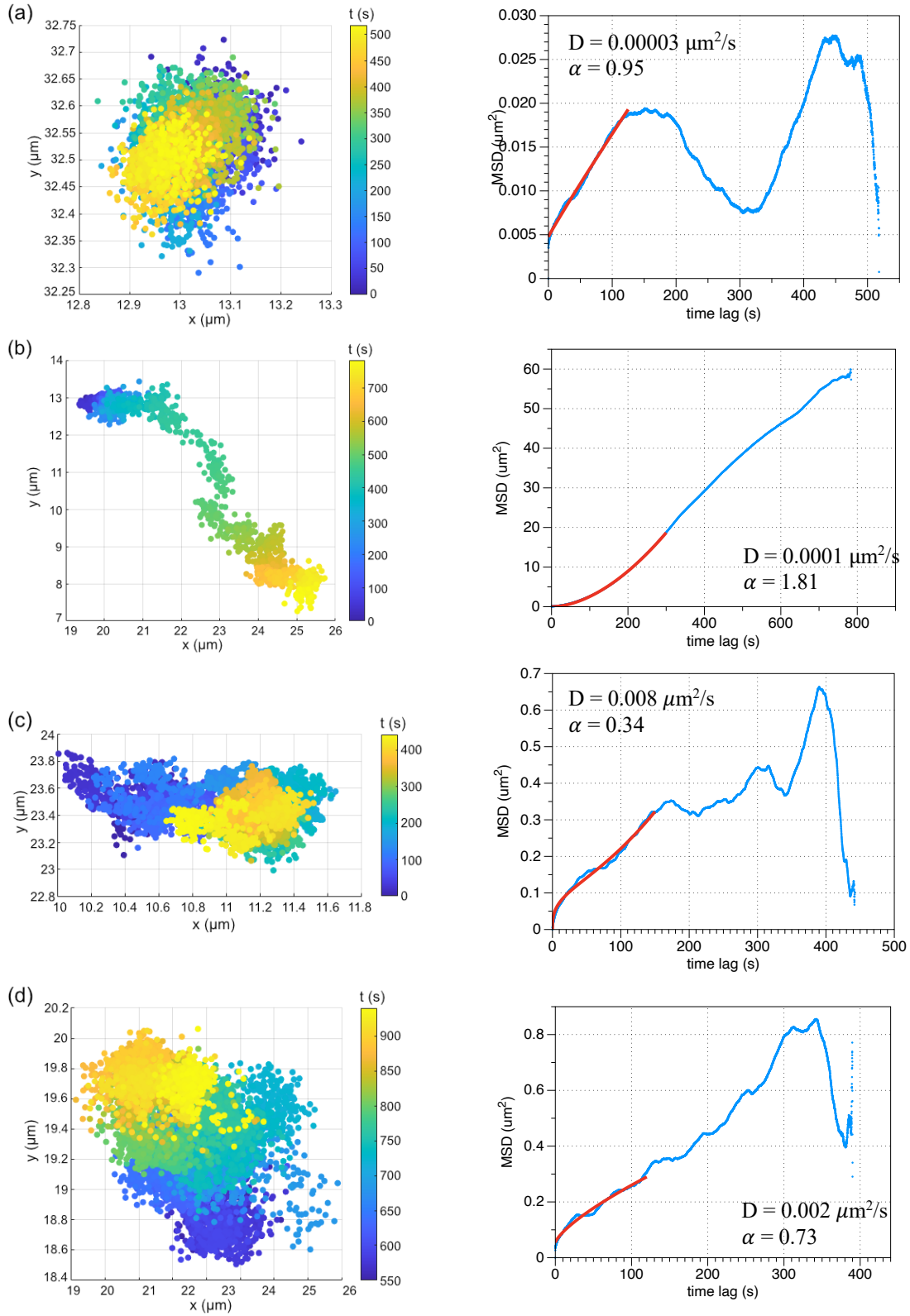


Figure 23: Representing trajectories and corresponding MSD curves of four 100 nm fND particles diffusing on top of the plasma membrane. The x-y coordinates correspond to the original detected image coordinates, the timescale is given by the gradient bar along the right of the plot. The mean square displacement (MSD) curve in time is plotted in blue, the red plot is the fit of the relevant part of the MSD curve used for the 2D diffusion coefficient  $D$  and  $\alpha$  parameter calculation.

The fNDs motion on top of the cell membrane was typically corralled diffusion. The corralls in this case are the membrane pits with receptors and concentrated charge. The fND gets trapped in such pits for substantial amount of time during its existence on top of the membrane. The

trajectory is often condensed in a small area, the particle usually travels a few micrometres in a large scale while the actual length of the trajectory ranges in hundreds of micrometres. Noticeable difference between the diffusion on top of the membrane and inside the cell was the speed of the fND. In average, the diffusion coefficient of fNDs on top of the membrane was ten times slower than of those inside the cell. That is, when excluding the active transport inside the cell which is not present on top of the membrane.

Typical examples of the 100 nm fND diffusion on the plasma membrane are in Figure 23.

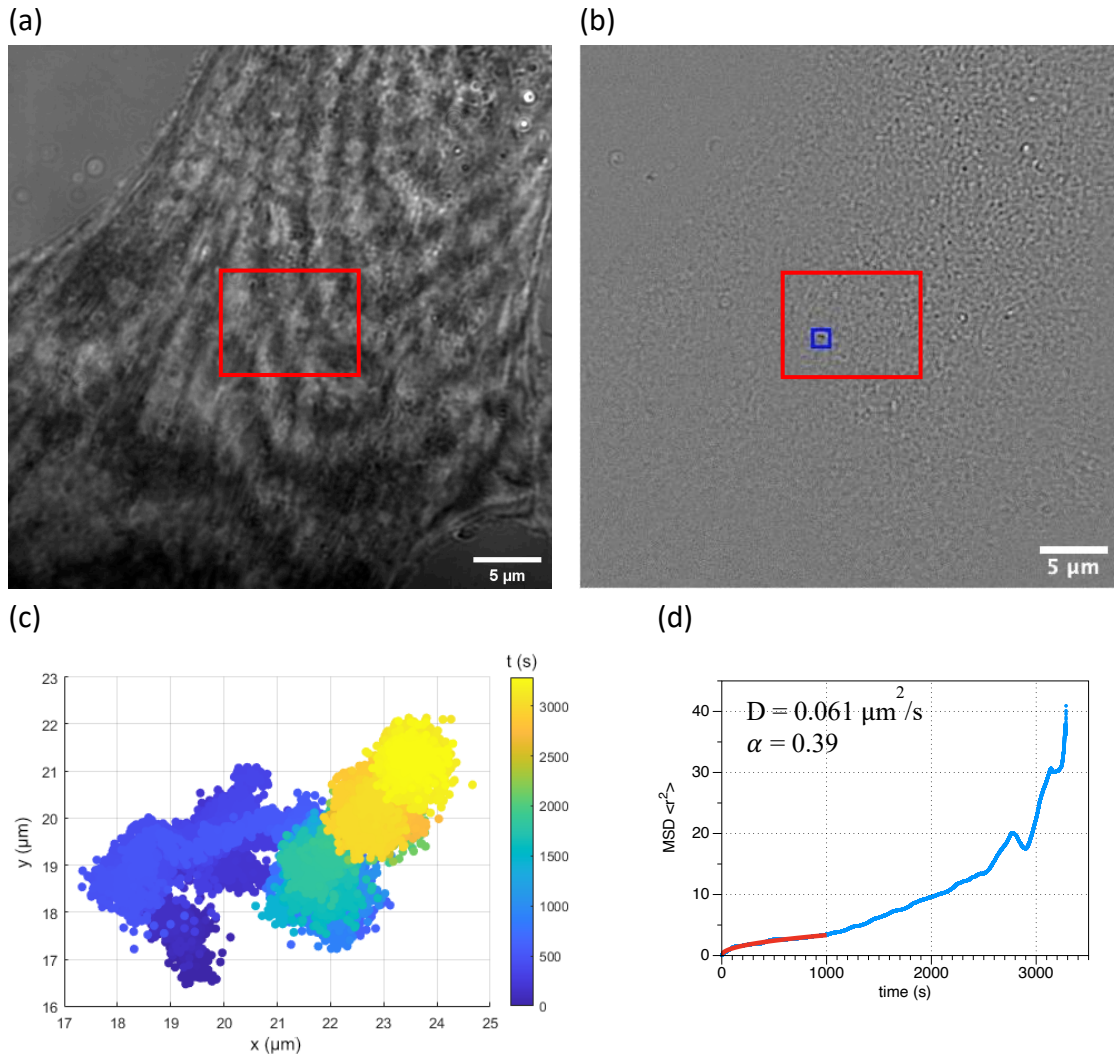


Figure 24: fND diffusing on the membrane of U2OS cell. (a) raw image of the recording, (b) filtered image with detected particle marked by a blue rectangle, the red rectangle marks the area displayed in the trajectory plot (c) The x-y coordinates in (c) correspond to the original detected image coordinates, the timescale is given by the gradient bar along the right of the plot., (d) corresponding MSD plot fitted by red line which was used for calculating 2D diffusion coefficient  $D$  and  $\alpha$  parameter.

### 5.5.1 Trajectory analysis

In case of complex way of motion where the particle undergoes different events along its trajectory, the spatial and temporal resolution is crucial. Depending on which information we want to acquire, we also need to choose the appropriate range or scale.

An example of a complex trajectory is in Figure 24 (c). The trajectory was recorded at a U2OS cell surface that was relatively flat, which can be assumed from the widely spread fringe pattern around the location of the red rectangle in Figure 24 (a). Despite being focused at the surface of the cell, recognized by the distinct fringe pattern created by the overall shape and slope of the cell surface, the cell dynamics is coming through also which makes the image very busy. To detect a single particle, a static and slow-moving structures were removed (Figure 24 (b)). Still, some cellular dynamics and fast-moving vesicles were left in the image but only fNDs on the cell surface were in the focus plane.

The whole trajectory was recorded for 58 minutes. The total trace length was 7134  $\mu\text{m}$ . Overall, according to the  $D$  and MSD curve shape, the diffusion would be classified as anomalous diffusion. To specify it better, we can consider  $\alpha$  parameter ( $\alpha = 0.39$ ) and we can call it an anomalous sub-diffusion. To learn more, the trajectory needs to be broken down to parts.

The original trajectory was broken down to four parts (A-D) displaying different behaviours. The timescale in Figure 25 - Figure 28 matches the timescale in Figure 24.

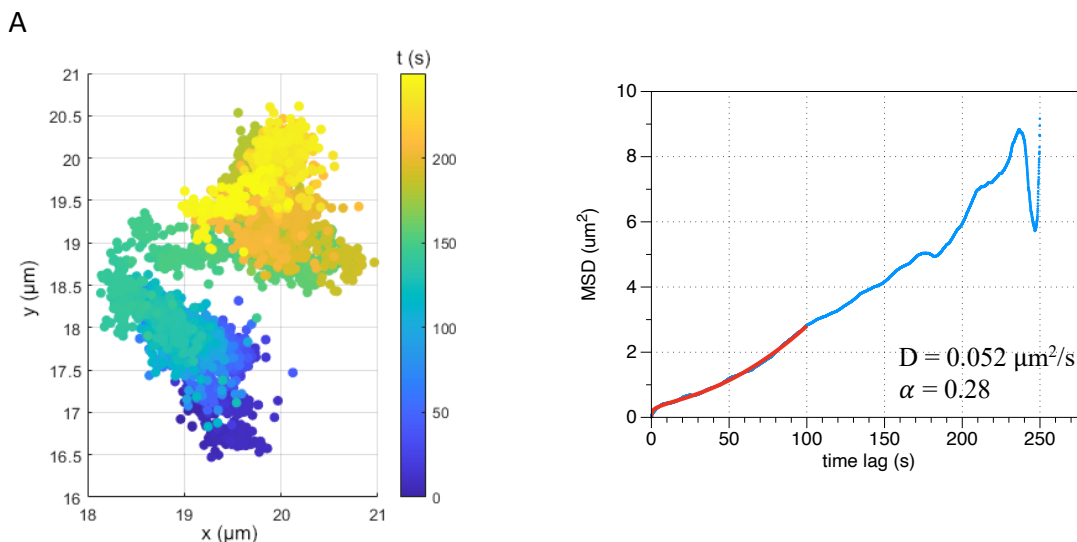


Figure 25: Segment A of the trajectory in Figure 24 (left) and corresponding MSD plot fitted by red line which was used for calculating 2D diffusion coefficient  $D$  and  $\alpha$  parameter (right).

The fND in the first segment A (Figure 25) was diffusing most sporadically and with highest speed ( $D = 52 \text{ nm}^2/\text{s}$ ). The MSD plot and its fitting show that the segment A was a case of anomalous sub-diffusion which was closer to normal diffusion than the other following

segments. In this case, it could be described as a fND that was not yet tightly attached to the plasma membrane by electrostatic forces. In this stage, the particle gets often detached from the cell and floats away.

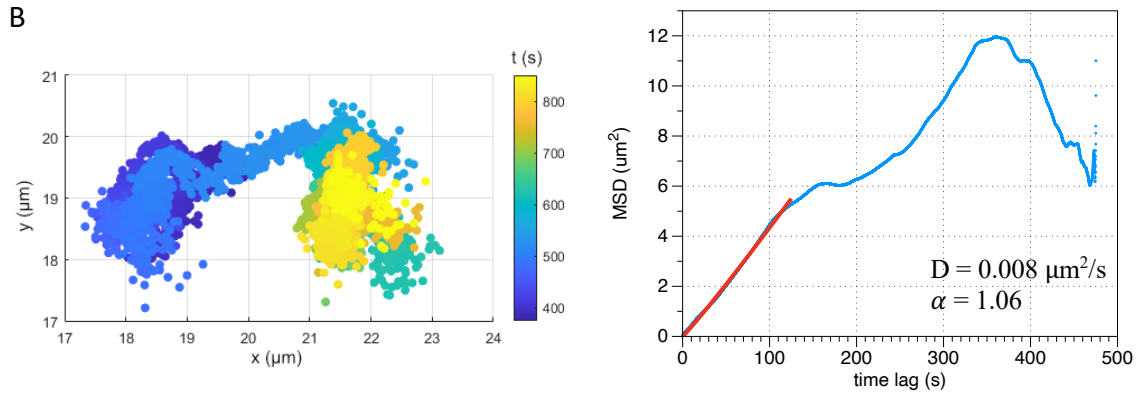


Figure 26: Segment B of the trajectory in Figure 24 (left) and corresponding MSD plot fitted by red line which was used for calculating 2D diffusion coefficient  $D$  and  $\alpha$  parameter (right).

In the segment B (Figure 26), the fND already diffuses with much slower speed ( $D = 8 \text{ nm}^2/\text{s}$ ), the fND copies the topography of the cell and the trajectory clearly shows two corrals, membrane pits, and passage in between them. When a particle is trapped in the membrane pit and the endocytosis process is not triggered the particle continues moving alongside the membrane and gets trapped in other pits until it eventually gets internalized or detached from the membrane. Segment B therefore exhibits the corralled diffusion.

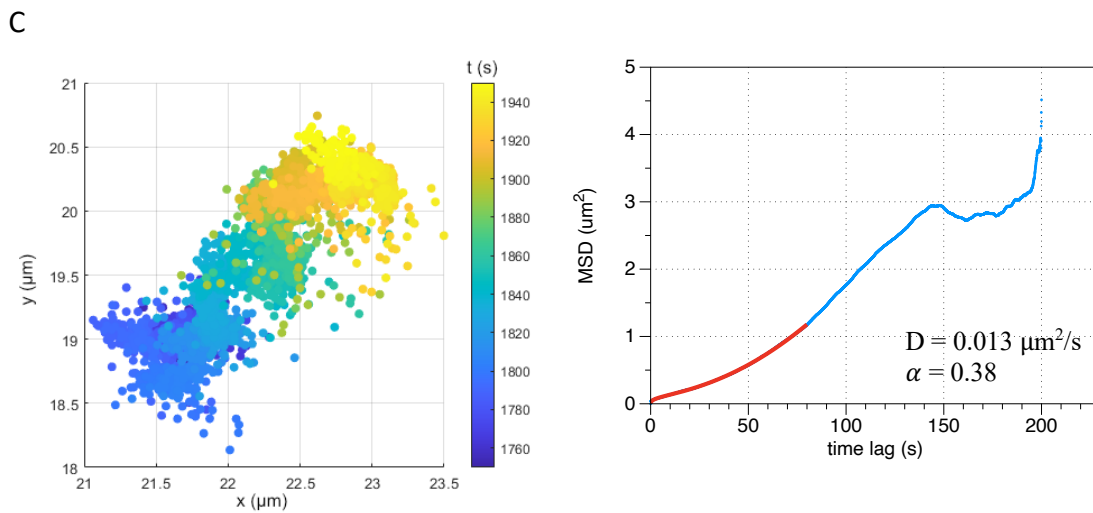


Figure 27: Segment C of the trajectory in Figure 24 (left) and corresponding MSD plot fitted by red line which was used for calculating 2D diffusion coefficient  $D$  and  $\alpha$  parameter (right).

In the segment C (Figure 27), the fND was moving in a directional motion through the crevasses in the plasma membrane which suggest both the wavy prolonged trajectory and the MSD plot curved upwards.

D

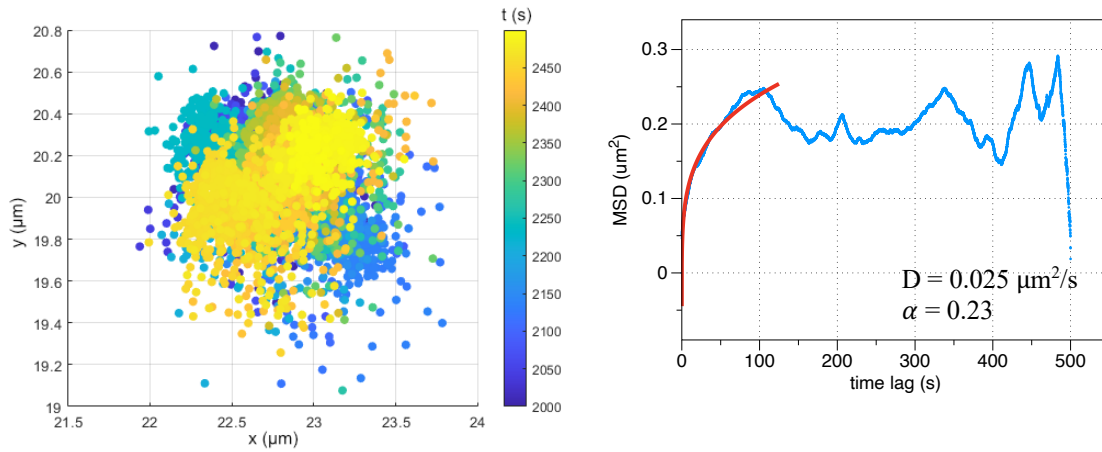


Figure 28: Segment D of the trajectory in Figure 24 (left) and corresponding MSD plot fitted by red line which was used for calculating 2D diffusion coefficient  $D$  and  $\alpha$  parameter (right).

The last segment D (Figure 28) is a clear example of confinement. Here we can see that the membrane pit is round and about  $1.5 \mu\text{m}$  large in diameter. Interestingly, the diffusion was faster inside the pit ( $D = 25 \text{ nm}^2/\text{s}$ ) than when diffusing along the membrane in the segment C or B.

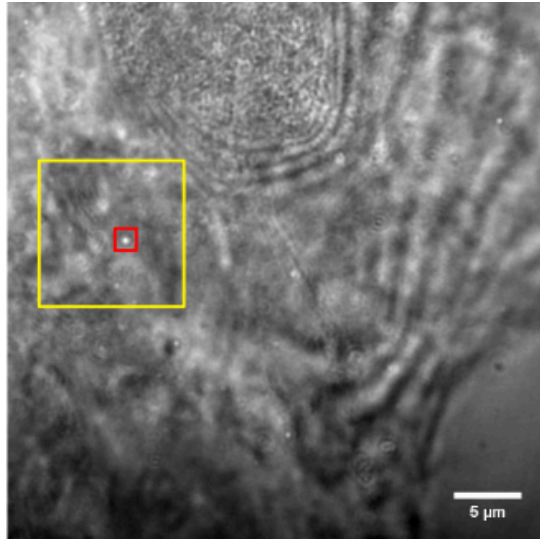
### 5.5.2 Interaction of PEG coated fND with plasma membrane

The internalization rate of PEG-fNDs proved to be faster than of the uncoated fNDs. Therefore, it can be assumed that the interaction with the plasma membrane will be different as well.

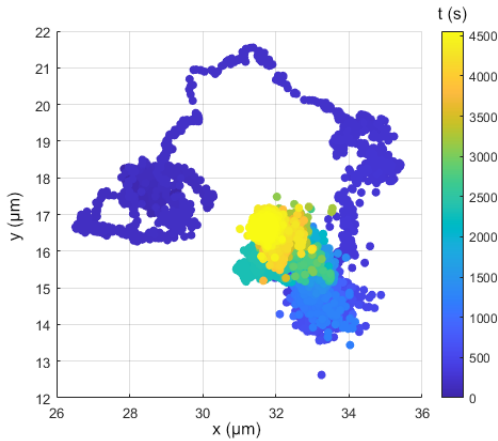
The same 100 nm PEG-fND sample as for the internalization experiment was used for tracking. The incubation time before observation was one hour in this case. The PEG-fNDs were observed from the moment of free diffusion in the culture medium until the moment of interaction with the plasma membrane to make sure that the PEG fND is not already internalized. Typical examples of PEG-fND and plasma membrane interaction are presented in Figure 29 and Figure 30.



(a)



(b)



(c)

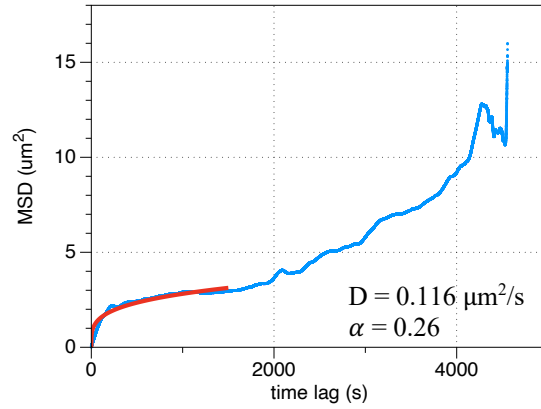


Figure 29: Tracking of PEG coated fND on U2OS cell membrane: (a) source recording with the tracked fND marked by red rectangle, yellow rectangle marks the area of the fND diffusion corresponding to the area in (b), (b) obtained trajectory after SPT, (c) MSD curve (blue) corresponding to the trajectory with fitting curve (red) and parameters  $D = 116 \text{ nm}^2/\text{s}$  and  $\alpha = 0.26$ .

The first example in Figure 29 shows trajectory with very fast diffusion at start and PEG-fND traveling long distance without much entrapment at membrane pits. Subsequently, it gets trapped in several pits in a row for the majority of the recording. As a result, both the trajectory and the MSD curve suggest confined diffusion. However, the difference from the bare fND diffusion is the fast almost free diffusion along the membrane captured at the beginning of the trajectory and consequently significantly higher diffusion coefficient  $D = 116 \text{ nm}^2/\text{s}$ .

The second example in Figure 30 captures another PEG-fND in a shorter timescale. This time the entrapment in any pit is not very significant, the particle travels along the membrane in a directional manner in the long timescale. The MSD curve suggests anomalous diffusion in this case. Additionally, the diffusion coefficient ( $D = 29 \text{ nm}^2/\text{s}$ ) is again higher than for bare fNDs.

(a)



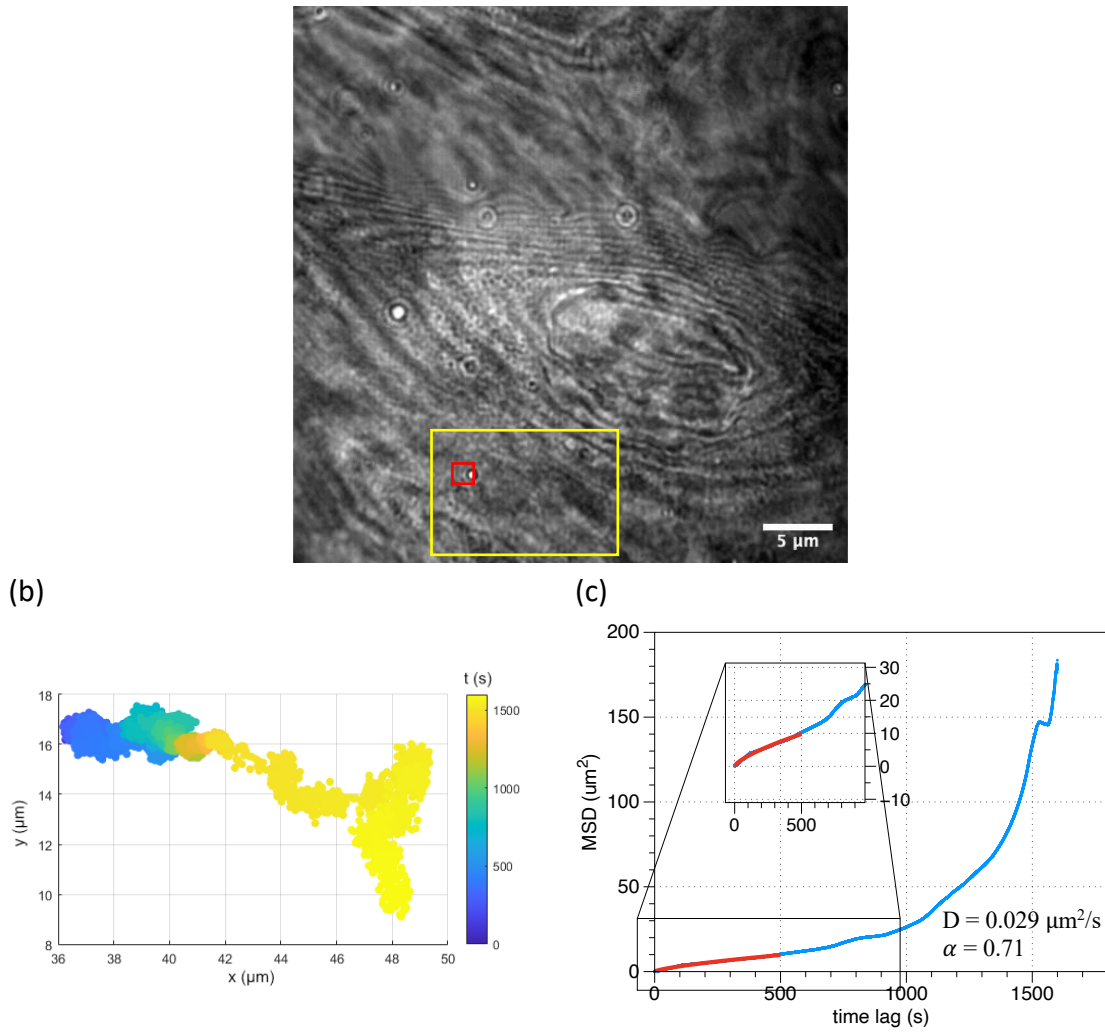


Figure 30: Tracking of PEG coated fND on U2OS cell membrane: (a) source recording with the tracked fND marked by red rectangle, yellow rectangle marks the area of the fND diffusion corresponding to the area in (b), (b) obtained trajectory after SPT, (c) MSD curve (blue) corresponding to the trajectory with fitting curve (red) and parameters  $D = 29 \text{ nm}^2/\text{s}$  and  $\alpha = 0.71$ .

## 5.6 Diffusion dynamics of natural cell vesicles

As a complementary experiment, the natural cell vesicles of U2OS cells were monitored. The general observations were that

- i) most of the vesicles are diffusing under the cell membrane and above the cell organelles,
- ii) vesicles are moving along the cell filaments or freely
- iii) their sizes range from tens to hundreds of nanometres and
- iv) there is higher number of vesicles in cellular cytosol than in the nucleus.

Several types of diffusion were observed: free, confined, and directional. All three types were detected in the cytosol where majority of vesicles had similar diffusion coefficient independent of the type of motion. The average 2D diffusion coefficient was  $D(\text{cytosol})_{\text{avg}} = 13.8 \text{ nm}^2/\text{s}$ . Near the nucleus, confined or directional diffusion was detected, again with similar diffusion coefficients  $D(\text{nucleus})_{\text{avg}} = 0.9 \text{ nm}^2/\text{s}$ .

Comparing these findings to the results obtained for fNDs, the vesicle diffusion closer to the 10 nm fND diffusion than 100 nm fNDs.

## **5.7 Conclusions**

As a promising technique for bioimaging, iSCAT was used to monitor the interaction between fNDs and living cells. The interaction was investigated for different sizes of fNDs, 100 nm and 10 nm, as well as different parts of the cell, membrane, cytosol and nucleus. First, it was observed that fNDs can be spontaneously internalized even by nonphagocytic cells like U2OS cells. Then, combining iSCAT and fluorescence detection enabled an unambiguous detection of very small particles down to size 10 nm and track them in real time on a long timescale. Based on these facts, the research proceeded to development of a tracking method and data evaluation.

The tracking in the cytosol showed differences between 10 and 100 nm fNDs. While 10 nm fNDs inclined more towards the free and confined diffusion, the 100 nm seemed to move notably faster and in more directional and confined motion. This signifies that particles of different size undergone different internalization mechanism to begin with and that larger fNDs are enclosed in vesicles whereas the smallest fNDs can diffuse through membrane without the need of active endocytosis process. Both sizes of fND also showed that they are able to enter the perinuclear space, however, they do not penetrate into the nucleus.

At the plasma membrane surface, all fNDs display corralled diffusion before entering the cell. Here, the importance of spatial and temporal resolution of SPT was demonstrated. Besides, the difference between bare and PEG coated fNDs interaction with plasma membrane was examined which revealed faster diffusion of PEG-fNDs along the membrane. Less affinity to irrelevant membrane pits of PEG-fND can be one of the reasons for the faster internalization rate of coated nanoparticles.

Lastly, the investigation of natural cell vesicles showed many similarities with fND diffusion in cytosol.

## 6 Thesis conclusions and outlook

This thesis presents a development of two consecutive methods, nanoparticle detection and characterization by axial profiling and theoretical modeling and nanoparticle tracking in living cells, as versatile tools for iSCAT microscopy. Those methods were then utilized for size characterization of fNDs that were used as probes for investigation of cell interaction with fNDs.

The size characterization by axial profiling consists of two parts. First is a practical part where the axial data of a single nanoparticle are collected by fluorescence combined iSCAT microscope that was especially upgraded for this purpose. The second part is the development of the theoretical model and data processing in MATLAB. In principle, this method uses the variation of scattering signal along the z focus range and fits the experimental data with a theoretical model. This enables to extract quantitative information about individual nanoparticles with nanometer accuracy. Additionally, considering it is a fully optical approach and compatible with aqueous environment, this technique stands out amongst the nanoparticle characterization techniques like TEM or AFM.[23]

This axial profiling technique can also be used for 3D bioimaging and tracking. Again, the investigation of fND-cell interaction was divided into two parts, the computational and the practical measurement. SPT software was written in MATLAB. The fluorescence combined iSCAT setup was modified for long-term measurement of living cells, methodology was established, and data collected. Finally, the trajectory data had to be analyzed to evaluate the fND diffusion behavior. The fND behavior and its differences inside and outside the cell are presented and discussed in great length in this work since there was many factors to unravel. Some of the main findings are that the large (100 nm) fNDs undergo an active endocytosis process and most often are transported in vesicles inside the cell. While the small (10 nm) fNDs enter the cell by passive uptake mechanism and diffuse without encapsulation inside the cell. However, both large and small fND travel in the direction of nucleus and both can enter the perinuclear area but not the nucleus itself. Additionally, the uptake rate and speed of diffusion was investigated for bare fNDs and functionalized fNDs. The results were also compared to the diffusion of natural cell vesicles that surprisingly resemble 10 nm fND diffusion in the cell. These findings can be useful in many areas of bioimaging or nanoparticle study, biosensors development, drug delivery or toxicity studies. One of the planned future projects is mapping the internal structure of a nucleus with 10 nm fNDs. This method opens new possibilities in mapping biological structures, it enables direct observation of the mechanism of endocytosis or other biological mechanisms and offers SPT with nanometric accuracy and long-term measurement in general.

The main goal of this thesis was to develop a rigorous method for characterization of fNDs and studying their interaction with living cells. This goal was fulfilled together with all the partial objectives, i.e., construction of fluorescence combined iSCAT microscope, development of theoretical model for nanoparticle characterization, establishing a method for SPT in living cell by iSCAT and studying the endocytosis of fND and its fate in the cell. In addition to that, correlation of fND fluorescence intensity with its size was investigated, the effect of fND functionalization on the interaction with cell was analyzed, cell structures were identified by iSCAT and behavior of natural cell vesicles were explored.

# List of publications

## Articles in journals

- [1] K. Žambochová, I.-B. Lee, J.-S. Park, S.-C. Hong, and M. Cho, ‘Axial profiling of interferometric scattering enables an accurate determination of nanoparticle size’, *Opt Express*, vol. 31, no. 6, p. 10101, Mar. 2023, doi: 10.1364/OE.480337.
- [2] I.-B. Lee, H.-M. Moon, J.-S. Park, K. Zambochova, S.-C. Hong, and M. Cho, ‘Three-dimensional interferometric scattering microscopy via remote focusing technique’, *Opt Lett*, vol. 45, no. 9, p. 2628, May 2020, doi: 10.1364/OL.386172.

## Conference contributions

### *Oral presentation + article in conference proceedings*

- [3] K. Žambochová, A. Patrochová, and D. Pánek, ‘Physical stability of small unilamellar vesicles probed by fluorescence correlation spectroscopy’. In *Instruments and Methods for Biology and Medicine 2017*, p. 30–37, Kladno, 2017.
- [4] K. Žambochová, J. Šimůnek, H. Sechovcová, H. and T. Jarošíková, ‘Proteomic approach to extracellular enzymes of anaerobic xylanolytic bacteria’. in *Instruments and Methods for Biology and Medicine 2015*, Kladno, 2015.

### *Poster presentation*

- [5] K. Žambochová, J.-S. Park, I.-B. Lee, S.-C. Hong, and M. Cho, ‘Diffusion dynamics of fluorescent nano-diamonds in living cells detected by fluorescence-combined iSCAT system’, in *IBS conference on Advanced Optical Imaging*, Seoul, Jun. 2019.

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## Summary

Single nanoparticle imaging, moreover, its quantitative characterisation or tracking have always been a challenge especially when using optical methods. Here, a fluorescence combined interferometric scattering (iSCAT) method in combination with theoretical modelling and data processing accomplishes all those challenges and offers a close insight into nanoparticle-cell interaction. In this work, iSCAT is used for size quantification of dielectric nanoparticles in range from 100 nm down to 10 nm. Although it is a fully optical method, the interferometric aspect allows to go beyond the diffraction limit. An axial three-dimensional profile of interferometric scattering from a single particle contains information about nanoparticle's exact location, its material properties or size. A theoretical model was then developed to unravel the detected scattering signal from a single particle to calculate its size. The size measurement was carried out with polystyrene beads and subsequently fluorescent nanodiamonds (fND). Characterized fNDs were used in further investigation of their interaction with living cells. This investigation uncovered different pathways of 10 nm and 100 nm fNDs during the uptake mechanism as well as their pathway inside the cell. A single particle tracking method with fluorescence combined iSCAT was developed along the way which allows to track even as small nanoparticles as 10 nm in long timescales up to several hours. Both introduced methods are versatile techniques offering future development and perspective in nanoparticle characterisation and optical bioimaging.