Examining Nanoparticle Interactions on a Cellular Scale Using Correlative Light Electron Microscopy

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Abstract: One key area of nanomedicine research is the mechanism of action of nanoparticles within the body, it’s biodistribution and pharmacokinetic properties. However, insight into the nanoparticle-cell interface is limited, due in part to the heterogeneous nature of nanoparticles as a drug class and the numerous possible interactions between cell and nanoparticle which stem from this diversity such as surface modifications, particle composition and final target tissue. Knowledge of the specific events which occur once a nanotherapeutic has reached its target are still very early in development; this is in part due to the difficulty of resolving interactions in sufficient spatiotemporal resolutions for a concrete understanding of the mechanism for nanoparticle-cellular uptake. However, this is crucially important for our understanding of the therapeutic capabilities of nanoconstructs as a whole. Traditionally, the study of nanoparticle-cell interactions has remained exclusively in either the light or electron microscopies, sacrificing either high resolution cellular information or accurate identification of specific biomolecules through fluorescent markers respectively. This study demonstrates a method for the study and categorization of nanoparticle-cell interactions using Correlative Light Electron Microscopy (CLEM), towards monitoring different drug delivery methods and their effect on target tissues at high resolutions.

Keywords: CLEM; focused ion beam-scanning electron microscopy (FIB-SEM); confocal microscopy; nanoparticle-cell interactions; transferrin-mediated uptake

1. Introduction

Recently, we are witnessing a sea-change in the design approach of novel therapeutics, we now recognize that in order to create more potent and less hazardous pharmaceuticals in the future, a more sophisticated approach is needed beyond conventional small-molecule therapeutics [1]. Medical research is beginning to explore different avenues of enquiry to discover more targeted drug delivery mechanisms. Accordingly, we need a modularized platform for delivering therapeutics in a targeted and consistent manner [2]. One such platform attempts to perform such a task through the careful design and presentation of therapeutics in 3-dimensions on the nanoscale similar to viral particles and other cellular machinery. It seems like a logical step to emulate biology’s own machinery for the purposes of defeating biological diseases [3,4].

Nanoparticles are a heterogeneous class of nanostructures with often very different physical and chemical properties from one another but are related through their characteristic size (<100 nm in at least 1 dimension) and the unique biophysical properties these size constraints illicit from these structures [5]. Unable to diffuse passively across cell membranes they lend themselves amenable to exploiting more complex receptor targeting pathways like endogenous processes utilized by our cells daily to grow, communicate and die. This higher level of abstraction allows for a variety of different targets and targeting designs [6]. However, current nanoparticle-based therapeutics have been very slow to make it to market and progress has been mired by the very properties we wish to exploit i.e. nanoparticle’s size and it’s 3-dimensional nature [7].

Currently, few therapies have been implemented clinically, therapies such as liposomes have had limited success and mainly perform an improved formulation role rather than an active agent for targeting disease [8]. Questions still remain to be answered in detail such as, how do nanoparticles behave on and inside cells and how can their behavior be modified to increase cell specificity, drug targeting, bio-availability or indeed how modification to one can affect the others? Research into nanoparticle uptake and bioprocessing tends to focus on two main areas, either through the use of fluorescent labels to track organelles/receptors [9,10] of interest relative to nanoparticles of different compositions or through the use of electron microscopic methods to study the exact position, number and morphology of organelles affected by nanoparticle uptake [11,12].

The aim of this study is to use a combination of both techniques, to create a symbiosis where the limitation of one method is mitigated by the other and vice versa. This correlated technique allows the pin-pointing of nanoparticle receptor interactions down to a couple of nanometers while also identifying the substituents with the accuracy of immuno- and transfected labels. This process can map the course of nanoparticle-cell interactions from receptor binding and endocytosis
to excretion and degradation.

To develop a method for correlative imaging of nanoparticle-cell interactions; this study focused on the endocytosis of the nanoparticles through the transferrin receptor. Endocytosis is characterized by the internalization of biomolecules into membrane-bound compartments. Vesicular trafficking is divided into two main categories, the clathrin-mediated endocytic pathway and the non-clathrin mediated endocytic pathway. The transferrin receptor (TIR) is a stereotypical example of the family of receptors internalized through the clathrin-mediated pathway. Overexpression of the TIR is characteristic of several different cancer cell populations [13], thus transferrin is a suitable candidate for drug delivery systems. Therefore, transferrin bound gold nanoparticles (Ti-AuNp) were used as a model particle for this study because of its safety, selectivity, tumor-targeting capabilities and because of its interest in the nanoparticle field [14].

Correlative Light Electron Microscopy (CLEM) combines both light microscopic techniques such as confocal microscopy and electron microscopic techniques such as FIB-SEM, these techniques complement each other, merging the large sampling volume and broad selection of fluorescent labels and applications e.g. Förster Resonance Energy Transfer (FRET) [15], Fluorescence Lifetime Imaging (FLIM) etc. with the high resolving power of the electron microscope. As nanoparticle-cell interactions occur on the scale of the tens of nanometers, combining both techniques provide unique insights greater than the sum of their parts, leveraging the discriminating power of light microscopy with the high precision and high spatial isotropic resolution of electron microscopy.

2. Materials and Methods

2.1. Subsection

A549 Human lung carcinoma epithelial cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in cell culture flasks (Stratagene, San Diego, CA, USA) with 10ml Complete Minimum Essential Media (cMEM) which contained Minimal Essential Medium (Corning Incorporated, Corning, NY, USA) with 10% Fetal Bovine Serum (FBS, Gibco, Ireland) and 1% Penicillin-Streptomycin (Pen-Stryp; Invitrogen, Carlsbad, CA, USA) in a humidified 37°C incubator with 5% CO2. Cells were passaged 2-3 times a week depending on the confluency of the cultures when passing i.e. between 60-90% coverage of flask surface, the medium was removed from the flasks and washed with Dulbecco’s Phosphate Buffered Saline (DPBS; Gibco, Carlsbad, CA, USA), the cells were incubated in 5ml of 0.05% trypsin-EDTA (Gibco) at 37°C for 3 min, the flasks were tapped gently to ensure complete detachment of cells from the flask surface. 7 mL of cMEM was added to the cell suspension and the solution was transferred to a sterile 50ml Falcon tube. The falcon tube was spun down at 200 g for 3 min and the supernatant removed. The cell pellet was resuspended in 10 ml cMEM and mixed thoroughly. 10 μL of the suspension was placed on a Neubauer haemocytometer and their concentration determined. A volume containing 5 × 10³ cells was mixed with 10ml cMEM in a fresh flask and incubated in an incubator at 37°C and 5% CO2. Cells were tested monthly for mycoplasma using the MycoAlert test kit (Lonza Inc., Morristown, NJ, USA).

2.2. Transferrin Absorbed Gold Nanoparticle Preparation

Gold Nanoparticles (100 nm) (Cytodiagnostics, Burlington, ON, USA) were mixed with holo-Transferrin protein powder obtained from Sigma-Aldrich to a final protein concentration of 1 mg/mL and incubated for 1hr at 37°C. The solutions were removed from the water bath and spun down three times at 400 g for 30 min, after each spin the supernatant was removed and replaced with an adequate volume of DPBS. The final nanoparticle solution concentration was measured using the Nanodrop Spectrometer 2000 and normalized to a concentration of 1 μg/mL at peak wavelength 572 nm and using the molar extinction coefficients supplied by Cytodiagnostics (1.57 × 10¹¹ M⁻¹ cm⁻¹). Particle stability, size and polydispersity was measured using the UNCLE Dynamic Light Scattering (DLS) instrument. The Z-average (Z) was calculated as the intensity-weighted mean hydrodynamic size of the particles in solution, derived from a Non-Negative Least Squares (NNLS) analysis of the measured correlation coefficient between the observed change in scattering intensity over time and that of a theoretical solution [16]. The PolyDispersity Index (PDI) was calculated as the square of the standard deviation (σ) divided by the square of the Z-Average (Equation.1).

\[ \text{PDI} = \frac{(\sigma/Z)^2}{1} \]  

PDI values < 0.05 are considered monodisperse i.e. of similar hydrodynamic diameter and those > 0.7 are considered to be polydisperse i.e. a mixture of different particle sizes [17].

2.3. Tf-Gold Addition to A549 Cells

A549 cells were split according to section 2.1 and 5 × 10⁴ cells were mixed with 3 mL of CMEM and added to each 35 mm culture dish with Gridded Coverslips (No. 1.5) (MatTek, Ashland, MA, USA). The dishes were incubated at 37°C with 5% CO₂ for 48 h. The media was removed from each dish and the cells were washed with 3ml of warm DPBS and 1ml of freshly prepared and normalized Tf-Gold nanoparticle solution, as per section 2.2, was added to each dish. The
cells were incubated at 37°C with 5% CO₂ for a period of time. The nanoparticle solution was removed and the cells were washed with 3 mL of cold DPBS (4°C) and a fixative solution of 1 mL of 0.2% (v/v) of glutaraldehyde (GA) and 4% paraformaldehyde (PFA) in DPBS was added, the dishes were placed in the fridge and incubated at 4°C for 1 hr. The fixative solution was mixed with 2 mL of cold DPBS and removed.

The cells were washed once more with 3 mL cold DPBS. For permeabilization experiments, a permeabilization solution of 3% (w/v) BSA and 0.1% (w/v) saponin in DPBS was added to the fixed cell and incubated at room temperature for 1 h. The cells were treated according to section 2.4 depending on the experiment.

2.4. Antibody Receptor Staining

Anti-Transferrin receptor rabbit IgG polyclonal antibody (1:100 in DPBS with 1% (w/v) Bovine Serum Albumin) (BSA; Abcam, Cambridge, UK) was added to each gridded coverslip and incubated at 4°C for 1 h or o/n. After incubation in primary antibody, the dish was washed with cold DPBS w/1%BSA and Anti-Rabbit IgG Alexa Fluor 647 antibody (1:200 in DPBS w/1%BSA) is added to the coverslip and incubated at 4°C for 1 h or overnight.

Anti-Clathrin heavy chain mouse IgG polyclonal antibody (1:200 in DPBS with BSA) (Thermo Fisher, Waltham, MA, USA) was added to each gridded coverslip and incubated at 4°C for 1 h or o/n. After incubation in primary antibody, the dish was washed with cold DPBS w/1%BSA and Anti-Mouse IgG Alexa Fluor 568 FluoroNanogold™ antibody (1:200 in DPBS w/1%BSA) (Nanoprobes, Yaphank, NY, USA) is added to the coverslip and incubated at 4°C for 1 h or overnight.

For double-stain experiments, the primary and secondary staining steps were performed sequentially with transferrin and anti-rabbit staining being performed first, as described above, followed by several washes of cold DPBS with 1% BSA and subsequent addition of the anti-clathrin and anti-mouse antibodies as described above. Depending on the experiment, 4’,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher) counterstaining was performed by adding 100 µL of 10 µg/mL solution to the cell and incubated in the dark for 1 min at room temperature and Phallodin Alexa Fluor 546 staining was performed by adding 5 µL of methanolic stock solution to 200 µL DPBS. This solution was added to the cells and incubated in the dark at room temperature for 20 min. After incubation the dish was washed with cold DPBS with 1% BSA, twice with cold (4°C) DPBS and finally suspended in cold DPBS. The dish was brought to the confocal microscopy core for imaging.

2.5. Confocal Imaging of Tf-Gold Incubated A549

Imaging was carried out with an inverted confocal laser scanning microscope in reflection mode, LSM780 (Carl Zeiss, Jena, Germany) using both 63 × 0.26 × 0.26 µm and 63 × 0.130 × 0.34 µm laser lines to image the nanoparticles, transferrin receptors and clathrin molecules respectively. The first image at ×10 (Image size: 512px × 512px / Pixel size: 1.66 µm × 1.66 µm (xy)) was to locate a ROI on the grid in widefield mode, second was a ×63 stack (Image size: 512px × 512px / Pixel size: 0.26 µm × 0.26 µm × 0.34 µm (xyz)) of the ROI with widefield, 488 nm and 561 nm or 633 nm laser lines to image the nanoparticles, transferrin receptors and clathrin molecules respectively. The third and final image was a ×2 digital zoom of a ×63 magnification (Image size: 512px × 512px / Pixel size: 0.130 µm × 0.130 µm × 0.34 µm (xyz)+) on a cell of interest. Several locations on the grid were taken in this manner.

2.6. Fixing, Staining and Resin Embedding of Confocal Imaged A549 with Tf-Gold Nanoparticles for FIB-SEM

This method was performed with some adaptations as previously described in [18]. Immediately after imaging the DPBS was removed and replaced with 2% Glutaraldehyde in 0.1 M Cocodylate Buffer (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) at 4°C, the cells were incubated at 4°C for 1 h.

For SEM visualisation of the FluoroNanogold™ antibodies, the cells were incubated 3 times (×3) with 50 mM glycine in PBS for 5 min each to remove trace aldehydes which inhibit gold enhancement. The cells were washed with 1% BSA in PBS for 5 min (×3) and finally for 5 min (×3) in ddH₂O. Using the Gold Enhance™ EM kit (Nanoprobes), 10 µL each of Solution A and B were mixed and incubated at room temperature for 5 min, 10 µL each of Solution C and D were added. The 40 µL were added to the cells and incubated for 5 min. The cells were washed with ddH₂O (×3).

After fixation and/or FluoroNanogold™ enhancement, the cells were washed with 0.1 M of cold cocodylate buffer
(×3) and incubated in 2% v/v OsO₄ in 0.1 M cacodylate buffer with 0.5% (w/v) Ferrocyanide (Thermo Fisher) for 1 h at room temperature (RT). The sample was washed with ddH₂O (×3) and incubated in cold-filtered 2% v/v Uranyl Acetate (UA) in ddH₂O at 4°C overnight. After 0/h incubation the UA solution was removed and the sample washed with ddH₂O (×3) and replaced with a 50 : 50 ethanol:water solution and incubated at RT for 5 min (×3). The ethanol : water solution was replaced with 70 : 30 ethanol : water solution and incubated at RT for 5mins (×3), this process was repeated for 90 : 10 ethanol : water (×3) and 100% ethanol solutions for 10 min (×2). During the dehydration procedure with ethanol the epoxy resin was prepared by creating “Solution A” by mixing DoDecenylsuccinic Anhydride (DDSA) with Embed-812 resin (EMS) to a ratio of 1 g : 0.76 g. Another solution, “Solution B” was also created by mixing resin hardener Methylene-5-Norbornene-2,3-dicarboxylic Anhydride (NMA) (EMS) with Embed-812 resin to a ratio of 0.87 g : 1 g.

10 min before addition to the cells, Solution A and B were mixed to a ratio of 2 mL : 8 mL and mixed thoroughly, immediately before addition to the cells the solution was mixed with 0.2 mL of accelerator Tris-(Dimethylaminomethyl) Phenol (DMP-30) (EMS) and heated to 60°C for 5 min to remove bubbles. After incubation in 100% Ethanol, a 50:50 Resin : Ethanol mixture was added to the cells and incubated for 1 h at RT and placed on an orbital shaker at < 5 Hz. The resin : ethanol mixture was replaced with 70 : 30 resin : ethanol mixture and incubated for 1 h at RT on an orbital shaker. This was repeated for 90 : 10 resin : ethanol and 100% resin solutions. After incubation in 100% resin for 1 h, a fresh batch of 100% resin solution was prepared as previously described and added to the cells. The cells were incubated at RT for 1 h and moved to an oven to cure overnight at 60°C.

2.7. FIB-SEM Imaging of Tf-Gold Incubated A549

The sample was mounted on an aluminum stub using a double-sided adhesive conductive carbon tab, and the sides painted with silver paint to prevent charge build-up, allowed to dry and then placed in a sputter coater (Cressington model 108), and coated with gold for 40 s at 30 mA.

After gold coating, the sample was placed into the sample chamber of the FIB-SEM. FIB-SEM imaging was performed using a Zeiss NVEision 40 microscope, with the SEM operated at 1.5 keV landing energy, a 60 μm aperture and backscattered electrons were recorded at an energy selective back-scattered electron (ESB) detector. The user interface employed ATLAS 3D from Carl Zeiss, consisting of a dual 16-bit scan generator assembly to simultaneously control both the FIB and SEM beams and dual signal acquisition inputs, as well as the necessary software and firmware to control the system.

The region of interest was located using the SEM, and the instrument was brought to eucentric and coincidence point at a specimen tilt of 54°, i.e. the specimen height where the specimen does not move laterally with a change in tilt and where the focal point of both FIB and SEM coincide. Once the exact milling area was determined with reference to the microscope images, a protective platinum pad was laid down on top of the area using a Gas Injection System (GIS) of size 60 μm × 30 μm and 5 μm in thickness. Alignment marks were etched into the platinum pad using an 80 pA FIB aperture to allow for automated tracking of milling progress, SEM focus and stigmatism during acquisition. After alignment etching, the platinum pad was covered with a carbon pad using the GIS to protect the etched marks from the milling process. After deposition of the carbon pad, a trench was dug using a 27 nA FIB aperture to allow for line-of-sight for the SEM ESB detector. After the trench was dug, the imaging face was polished using a 13 nA FIB aperture. The FIB aperture was changed to 700 pA and SEM imaging area selected (Typical Image size: 4000 px × 2000 px /Pixel size: 15 × 15 × 15 nm³ (xyz) the automated acquisition software was set up and run until all the sample area was acquired.

2.8. Electron Dispersive X-Ray Spectroscopy

EDS detection was performed on a FEI Helios 660 Nanolab FIB-SEM with a 5keV landing energy, a 200pA aperture and a windowless Oxford XMax Xtreme detector. Points of interest were selected for analysis and spectra were acquired from 30 sec exposures at a time. The data was graphed using DTSA-II software (version Jupiter, National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA).

2.9. Image Processing and Segmentation

2.9.1. Confocal Image Processing

Confocal image file (.lsm) was opened in ImageJ and the channels were split (Red:568/647/Green:488/Widefield). The coverslip bottom was found using the green laser line reflectance and everything below this Z-height was removed. Each image was set to a 10-bit grayvalue (images were acquired at 12-bit) for both the Red and the Green channels. Both channels were merged together, saved as .tif and used for correlative alignment.

2.9.2. FIB-SEM Image Processing

After SEM acquisition the individual image files (.tif) were aligned using IMOD’s tiltxcorr program and binned by 3 in the x and y planes to produce isotropic pixels of size 15 nm × 15 nm × 15 nm (xyz). The images were opened in
ImageJ and cropped to isolate ROIs, a representative slice was picked and an area of size $10 \times 15 \, \mu m$ was selected and using the “Enhance Contrast” function was used to normalize and equalize the whole stack relative to the chosen area. The stack was binned by 2 in the x,y and z axes to produce a final voxel size of $30 \, \mu m^3$; the final stack was resliced down the Y axis to aid in manual alignment and registration of the light and electron images.

2.9.3. Semi-Automated Registration, Alignment and Correlation of Light/Electron Images

Both Confocal and FIB-SEM stacks were moved to Icy software [19] where both stacks were opened in the eC-CLEM plugin [20] and gold nanoparticles in both images were correlated. The eC-CLEM software aligned the stacks based on the coordinates of the shared registration marks. A non-rigid transformation was also performed on alignments that didn’t correlate efficiently, this inefficient correlation is mainly due to the contraction of cells after dehydration and resin embedding.

2.9.4. Segmentation and 3D representation of Cellular Structures

The correlated data set allowed for identification of nanoparticles of interest, i.e. nanoparticle/Transferrin receptor co-localisation. These nanoparticles of interest were selected in a high-resolution image stack (Voxel size: $5 \, \text{nm} \times 5 \, \text{nm} \times 15 \, \text{nm}$) and cropped in volumes of $2 \, \mu m^3$. These volumes were moved to Slicer-3D software [21] for segmentation. Cellular structures of interest i.e. cell membrane, vacuoles and nanoparticles were manually segmented by using threshold values appropriate for the structures and using automated segmentation methods described in [22].

3. Results

3.1. Nanoparticle Characterisation and Stability

In order to ensure a uniform particle preparation and the stability of prepared nanoparticles in solution during nanoparticle uptake experiments, it was necessary to analyse the particle size and distribution over time in solution. Table 1 demonstrates a shift in hydrodynamic size ($Z$-Average), measured by Dynamic Light Scattering (DLS) of 100 nm gold particle upon Transferrin absorption. The polydispersity index is used as an indication of the relative distribution of particle sizes within the measured population.

| Sample                                | Size – $Z$-Average (nm) (n=4) | PDI (n=4) |
|---------------------------------------|-------------------------------|-----------|
| Bare Gold (100nm) in 1% (v/v) PBS in Water | 92.6                          | 0.05      |
| Transferrin Absorbed Gold in PBS      | 117.2                         | 0.15      |
| Transferrin Protein in PBS            | 3.5                           | 0.85      |

1n=number of technical replicates

Figure 1a, demonstrates the increase in hydrodynamic diameter upon addition of transferrin to the solution and the broadening of the peak reflects the increase in the polydispersity of the solution. Figure 1b, indicates that the prepared transferrin particle solution remained stable up to 15 h. The gradual increase in $Z$-average and subsequent drop over the next 8hrs is most likely due to the gold nanoparticle agglomeration typical of nanoparticles of this size and not aggregation, nevertheless this graph shows that the particles are stable well beyond the timescale of nanoparticle passaging in this project (< 4 h) and no passage of gold nanoparticles was made 12 h after production.
Figure 1. DLS Analysis of Transferrin Bound Nanoparticle Stability. a) DLS intensity measurement of 100 nm Gold particles before (orange) and after (blue) transferrin absorption. b) DLS Z-Average stability measurement of Tf-absorbed gold nanoparticles at 37°C over a 23 h period.

3.2. Characterization of Nanoparticles and Heavy-Metal Staining in FIB-SEM Images

Gold nanoparticles were chosen for this FIB-SEM analysis, primarily due to their high electron-scattering properties which generated high-contrast objects, easily identifiable in the acquired FIB-SEM volume. However, due to the application of heavy metal contrast agents there were a number of high contrast objects in our acquired volumes which required identification in order to ensure accurate characterization of gold nanoparticles.

Energy-Dispersive X-Ray Spectroscopy (EDS) analysis allowed for the elemental characterisation of the three main components of the sample with high electron-scattering intensities (circled) and allowed for the verification of gold nanoparticle size and shape in the cell sample, for future acquisitions. The three main elements of interest in Figure 2 are osmium, gold and uranium, characteristic peaks of each element in the EDS spectra were circled green, red and blue respectively. Elemental chlorine was found to varying degrees in all datasets due to the potassium and sodium chloride solutions used in PBS and cell culture media, carbon and oxygen peaks were also present. Gallium peaks were present to varying degrees in all datasets, due to the use of focused gallium ions to ablate the surface of the sample in standard FIB-SEM acquisition.
Figure 2. Energy-Dispersive X-Ray Spectroscopy (EDS) Analysis of Resin Block. a) SEM image of typical central slice through nanoparticle treated cell, with high-scattering ROI’s selected for EDS analysis circled; b) Relative abundances of key elements in EDS spectra. Scale bar: 1 μm.

The first object in the volumes to be analysed (Figure 2a), circled blue, was found to almost exclusively contain uranium (Figure 2b), this is a common feature of uranyl acetate solutions which can develop aggregations such as the one present here during specimen preparation. The second object investigated in these volumes, the putative gold nanoparticle, circled red, was confirmed to indeed contain high concentrations of elemental gold. The relatively low number of counts compared to the other two spectra is due to the small nanoparticle size, which is quickly ablated by the intense electron beam. Finally, circled in green, was found to contain high concentrations of osmium with trace amounts of gold and uranium. The identification of osmium as the primary source of the high electron-scattering found in these types of cellular vesicles, indicate the presence of lipids, when coupled with their relatively large size (~500 nm) suggests these vesicles are lysosomes and not intracellular accumulations of gold nanoparticles.

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3.3. Determination of Fixation and Permeabilization Parameters for Retention of Ultrastructural Information from Light to Electron Microscopy

During CLEM analysis it became apparent there was a need for optimisation of cell preparations for electron microscopy. Paraformaldehyde fixation preceding permeabilization was not sufficient to preserve cellular ultrastructure during the harsh dehydration and resin embedding steps necessary for EM acquisition (Figure 3a).
Figure 3. Comparison of Ultrastructural Features of A549 Human Lung Cell Carcinoma Cells Before (a) and After (b) Optimisation. Fixation and permeabilization conditions for a) 4% v/v PFA fixation for 30 mins followed by 1 h incubation in 0.5% w/v Saponin with 1% BSA at RT and for b) 0.2% v/v GA and 4% v/v PFA fixation followed by 1 h incubation in 0.1 w/v Saponin with 3% BSA at 4°C.

GA fixation was avoided in early experiments due to its associated reduction of antigenicity and autofluorescence in Light Microscopy (LM), however after testing several conditions it was determined that GA was necessary to stabilise cellular structure enough to survive permeabilization and subsequent dehydration and embedding. A concentration of 0.2% v/v of GA, in combination with a reduced saponin concentration (0.1% w/v) was determined to be optimal to allow for sufficient retention of high-resolution ultrastructural information in the EM (Figure 3b) while also minimising undesirable effects in the LM.

3.4. Correlative Light Electron Microscopy Analysis of Nanoparticle-Cell Interactions

Figure 4 illustrates the steps required to move from light microscopy to electron microscopy with the help of gridded coverslips. By co-localising nanoparticle signal with that of the transferrin receptor it was possible to focus the ultrastructural analysis of nanoparticle uptake on specific receptor-mediated interactions, rather than analysing several of specific and non-specific uptake mechanisms.
image with co-localized signals (pink circle) of nanoparticles (green) with transferrin receptor (red) and nucleus (blue);

e) SEM image of resin surface with same region of interest as in Figure 4a,b;
d) Maximum Intensity Projection (MIP) of FIB-SEM volume with the same co-localized nanoparticles as in Figure 4b (pink circle);
e) MIP of Correlated Light Electron Volume.

Correlative 3D projection of single nanoparticle with colocalized light microscopy signal.

Over the course of several experiments a number of common structural components were observed between cells. Figure 5 contains exemplar images of the types of structures associated with nanoparticle receptor mediated uptake.
Figure 5. Segmented Images of Different Stages of Nanoparticle Uptake. (i) High resolution EM slices with manually segmented (Pixel size: 5 × 5 nm (xy); 5 × 15 (xz) / Scale bar: 500 nm); (ii) High resolution EM with correlated LM slice (Red: TfR / Green: AuNp / Cyan: Nucleus / Blue: Clathrin) (Pixel size: 5 × 5 nm (xy) / Scale bar: 500 nm); (iii) Segmented 3D volume of EM data (Pink: Cell Membrane/ Green: AuNp/ Blue: Low UA/OsO4 labelling density (e.g. Vacuoles)/ Red: High UA/OsO4 labelling density (e.g. high lipid/protein content)/ Yellow: Lysosome) (Voxel size: 5 × 5 × 15 nm³ (xyz) / Scale bar: 500 nm). a) Np-Tf Receptor (TfR) co-localised on cellular extension: Nanoparticles bind to transferrin
receptors on cellular protrusions.; b) Np-TfR co-localised at cell surface: Nanoparticles can also bind receptors on the surface of cells, but it is difficult to determine whether this is a result of the retraction of cellular protrusions or a separate cellular event. Some examples have indicated the presence of microtubules consistent with the retraction of cilia from the surface of the cell, whilst others are absent.; c) Np transport in endocytic vesicle: This shows the nanoparticle completely engulfed by a cell in an endocytic vesicle; d) Np-TfR co-localised engulfing of Np: The nanoparticle is engulfed in a pocket of cellular membrane at the base of a cilium before uptake is completed and is connected to an early endosome.; e) Np-TfR co-localised in an early endosome: As the nanoparticle is transported deeper into the cytosol, it fuses with an early endosome for sorting and further trafficking.; f) Np in a Late Endosome/Multi-vesicular body (MVB): In later stages of nanoparticle trafficking the nanoparticle is transported to a late endosome in preparation for degradation and exocytosis.; g) Lysosomal fusion and formation of Autolysosome: The nanoparticle is degraded further in a lysosomal vesicle within the autolysosome.; h) Particle Excretion: After trafficking through the cell the nanoparticle is excreted from the cell.

Vesicles in EM volumes were classified according to previous morphological studies of intracellular compartments, as follows [23–25]:

Endocytic vesicles: Small spheroid vesicles (50–200 nm diameter) with densely labelled membranes and an inner vacuole.

Early endosomes: Large irregular vesicles (200–400 nm diameter) with densely labelled reticulated membranes and compartments with an inner vacuole.

Late endosome/Multi-vesicular bodies: Large spherical vesicles (400–700 nm diameter) containing several smaller (20–100 nm) vesicles.

Lysosome: Large (400–700 nm) very densely labelled spherical vesicles with no visible inner compartments.

Autolysosome: Extremely large (0.7–1.5 μm) vesicle containing multiple compartments of varying labelling density and containing at least one lysosome.

4. Discussion

The study of nanotherapeutics is particularly suited to the use of correlative light electron microscopic methods. This manuscript presents methods for assessing the cell’s behaviour and interactions which occur in nanoparticle-cell interactions and demonstrates the ability to track multiple biochemical tags of interest to high resolutions and determine the structural environment nanoparticles encounter along their path through the cell.

This investigation generated a correlated 3D map of a model nanoparticle system’s interaction throughout the cell, using both light and electron information to identify the key checkpoints from initial binding to final excretion of the nanoparticle. It was noticed early on that sample preparation methods are often designed explicitly for one mode of microscopy, in ways that are regularly to the exclusion of another, e.g. fixation methods that work best for EM samples often degrade protein antigenicity, making fluorescent labelling difficult. Therefore, this method, which retains both the ultrastructural and antigenic character of the specimens throughout the light and electron microscopy processes will be extremely useful in studying nanoparticle action in cells in the future. Although this method relies on gold nanoparticles as both markers for correlation (due to their reflective and opaque properties in both light and electron microscopies respectively) and as subjects themselves for study, this method can be applied to a wide variety of nanoparticles with the use of standard fiducial markers to correlate the two microscopies.

Despite several advantages of this methodology over conventional imaging in either light or electron modes, there still remains a number of limitations to this technology. The principle limitation is that sample preparation in either imaging mode must not preclude the ability to image in the other, for most applications this can be avoided by careful experimental design, however this does limit the number of conditions and types of experiments available to CLEM techniques. Another common issue with CLEM methods is the limited quantitative interpretability of EM datasets [26], however this is a very active field in electron microscopy and computational biology and recent advances [22,27,28] are developing methods for both the accurate segmentation and quantitation of EM datasets. Finally, probably the largest hurdle to the broad application of this technology to the wider scientific community, is the relatively high instrument cost and maintenance fees of the FIB-SEM, not to mention the infrastructure required to house these instruments and to handle/process the large files produced by these microscopes. However, just as the recent cryo-electron microscopy revolution [29,30] has led to a surge in the adoption of transmission electron microscopes and witnessed a reduction in the cost of producing high quality protein structures [31,32].

In conclusion, this technique can be applied to a wide variety of nanotherapeutic studies and expanded to even higher-resolution techniques such as STTochastic Optical Reconstruction Microscopy (STORM) to identify and differentiate smaller cellular structures in electron microscopy [33], often key in the accurate targeting and distribution of therapeutic agents. This manuscript demonstrates a proof-of-concept, that defines the biomolecular makeup of nanoparticle-cell interactions on a nanoparticle by nanoparticle scale.

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