Studying NP uptake into cells

Advanced training in understanding the safety of nanomaterials

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Burgos 2017

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Background

- Environmental nanoscience group / University of Birmingham
  - Birmingham co-ordinate several EU funded projects and involved in many more (NanoMILE, NanoGenTools, NanoDefine Qnano, NanoValid, NanoCommons, FutureNanoNeeds, NanoFase, ACENano, MARINA, NERC projects)
- Facility for Environmental Nanoscience Analysis and Characterization (FENAC)
  - State of the art analytical facility
  - Support research and offer services for academia and industry
- Birmingham Advanced Light Microscopy facility (BALM)
- Multidisciplinary doctoral training centres
  - Incorporate projects based on NP synthesis, surface modification, imaging and analysis

UNIVERSITY OF BIRMINGHAM

ACEnano
Analytical and Characterisation Excellence

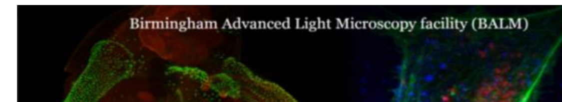
FENAC
FACILITY FOR ENVIRONMENTAL NANOSCIENCE ANALYSIS AND CHARACTERISATION

NERC SCIENCE OF THE ENVIRONMENT

NanoMILE

Northwestern University

EPSRC
Pioneering research and skills



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Nanoparticle introduction

NANOGENTOOLS

- 3 dimensions <100 nm
- Different properties to bulk material
- Increase in use in commercial and biomedical applications
- Lack of information on their release and subsequent effects and therefore difficulty in hazard / risk identification

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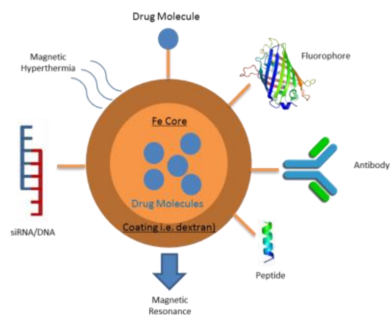
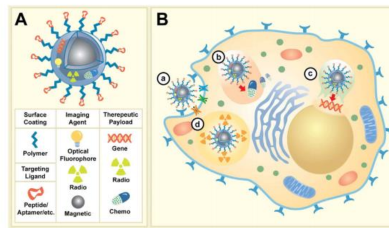
Nanoparticles in biomedicine

NANOGENTOOLS

- NPs have useful properties that can be manipulated
  - Small surface area to volume ratio
  - Modifiable surface
  - E.g. SPIONS: MRI, Drug Delivery, Hyperthermia treatment
- Although they show promise, NPs often lack efficacy in the clinic
  - Lack of linking physical properties, and particle enviornment with uptake and effects

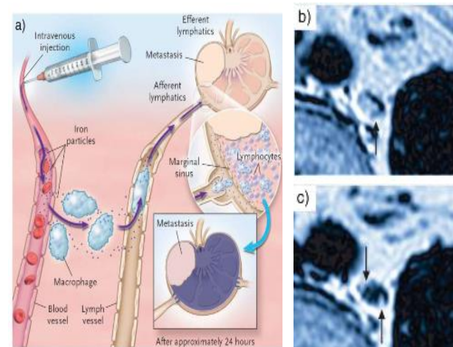
Drug delivery

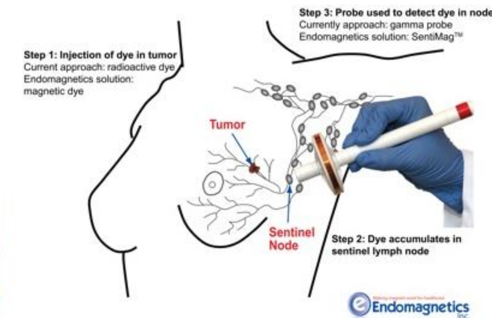




Veiseh *et al*, 2010

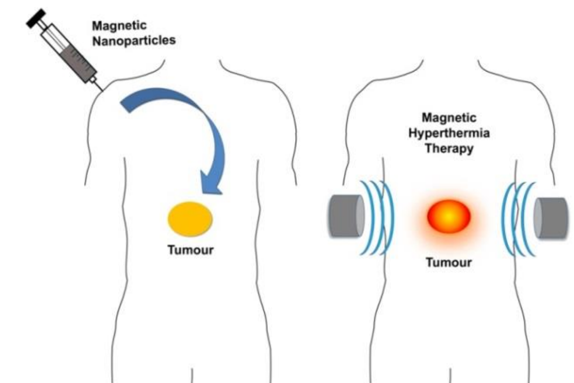
Diagnostics





Memarasadeghi *et al*, 2006

Magnetic Hyperthermia



Andrade *et al*, 2011

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Problems with NP use

- Nanoparticles show a lot of promise for applications but often fail to perform in the clinic

- Lack of understanding of the cellular interactions following NP exposure

- Lack of cheap, widely available high throughput methods for investigation

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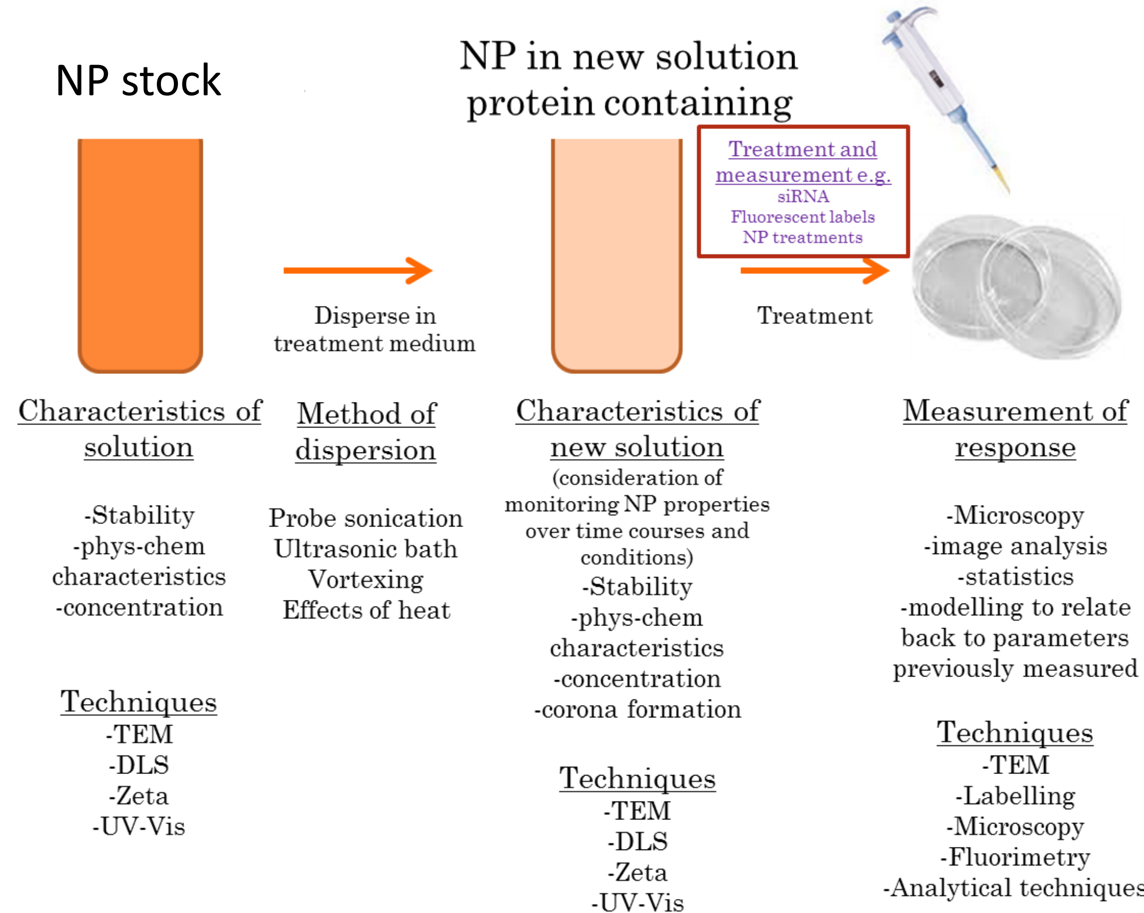
Experimental design in cellular studies

Biological experiments with nanoparticles

NANOGENTOOLS

- Characterization methods
- Dispersion (and characterization)
- Dosing considerations
- Cell type and exposure configuration
- Imaging method / end point
- Quantification of images



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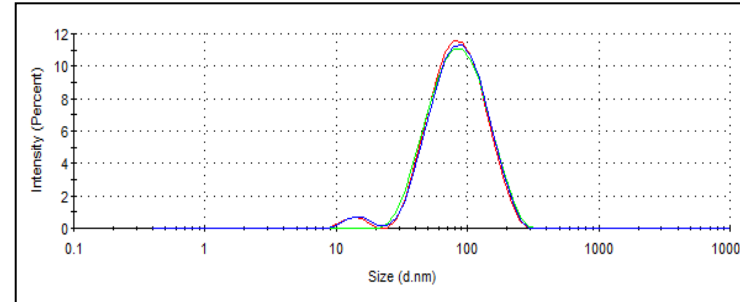
Biological experiments with nanoparticles

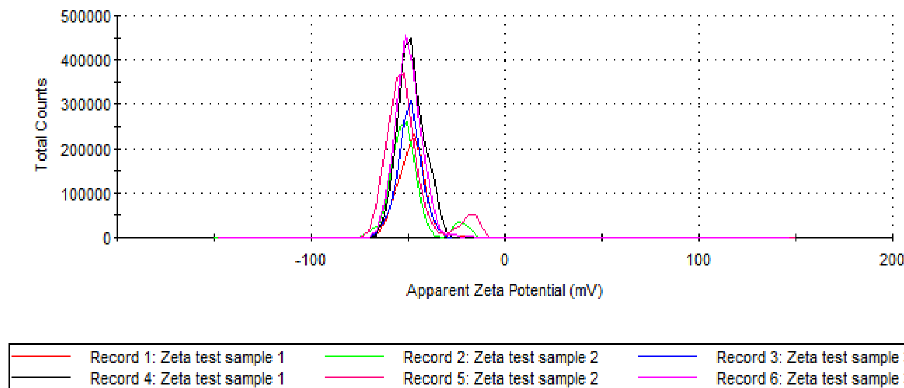
NANOGENTOOLS

- <u>Characterization of NP stock</u>
  - Size distribution, PDI, agglomeration state
  - Surface charge
  - TEM, DLS, DCS, FFF, STEM, FCS, XRD,
- Dispersion
- Dosing considerations
- Cell type and exposure configuration
- Imaging method

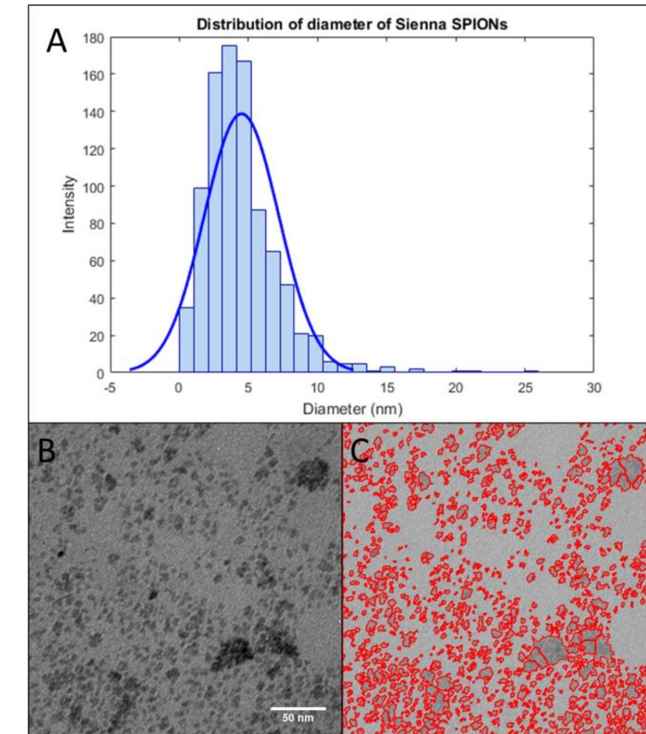
DLS: Size distribution



Zeta potential



TEM: Core size distribution



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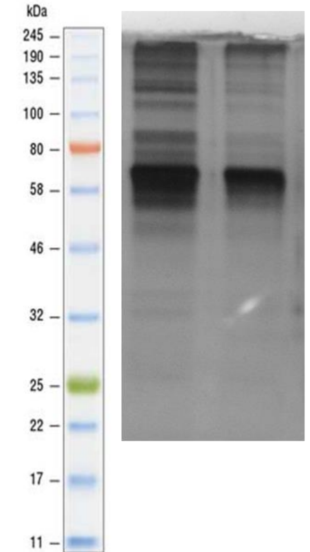
Biological experiments with nanoparticles

NANOGENTOOLS

- Characterization methods
- <u>Dispersion</u>
  - Sonication type
  - Optimize energy
  - Characterized in exposure media (DLS, TEM, Corona, Over time)
  - Stability
- Dosing considerations
- Cell type and exposure configuration
- Imaging method

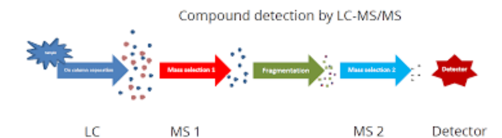
Sonication method

Corona characterization



Characterize the dispersion for key properties:

- Stability of size and zeta potential over time
- Protein binding (corona proteins) (CE-MS / PAGE)
- Size - TEM



Proteomics

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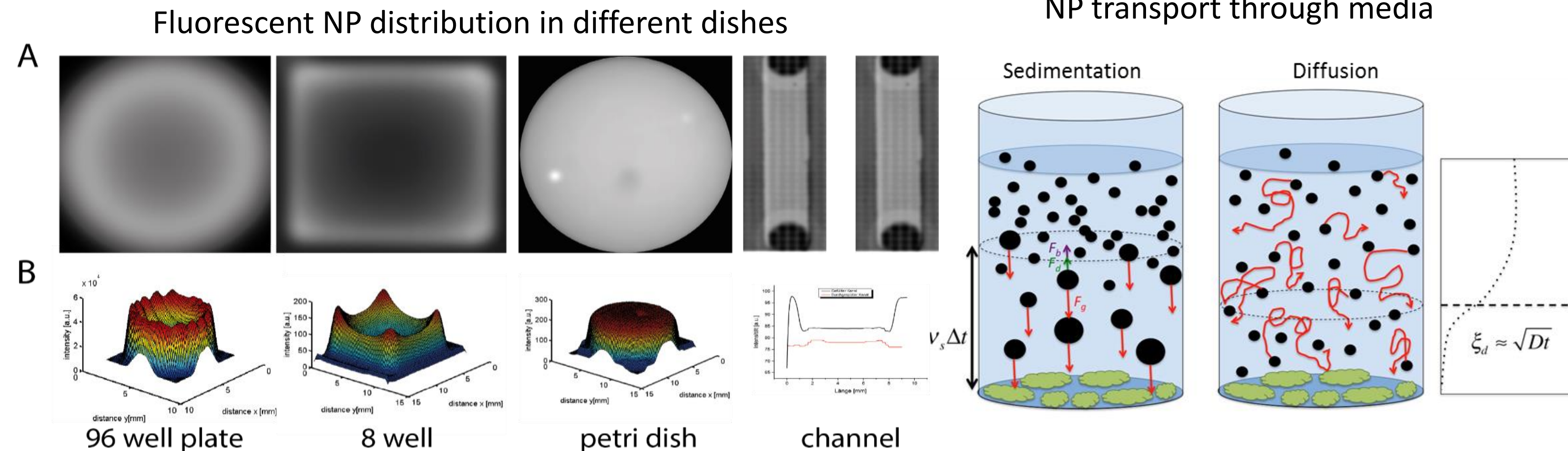
Biological experiments with nanoparticles

NANOGENTOOLS

- Characterization methods
- Dispersion
- <u>Dosing considerations</u>
  - Dose metric
  - Global Vs Local dose
  - Loss of dose to container and container effects
  - Sedimentation and diffusion – modelling
  - Agglomeration over time (characterizations)
- Cell type and exposure configuration
- Imaging method



Manuscript under review

<u>Record all of the experimental metadata</u>

- Well format (6, 12, 24, 96)
- Media volume
- Concentration in particle number and mass per surface area

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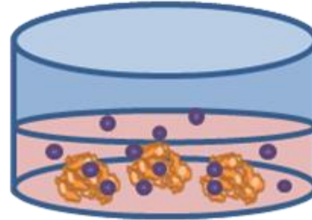
Biological experiments with nanoparticles

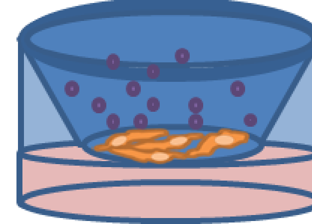
NANOGENTOOLS

- Characterization methods
- Dispersion
- Dosing considerations
- Cell type and exposure configuration
  - What process is being modelled?
  - Simple method or physiologically relevant?
  - Control of dose or model dose
  - Transport of material in system
- Imaging method

Cell spheroid cultures

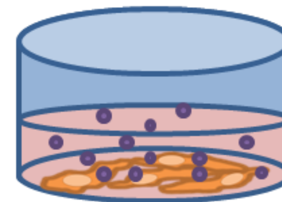


Air-liquid interface exposure

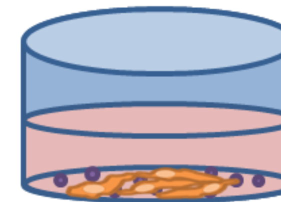


- Submerged 2D
- Inverted 2D
- NP surface
- Air-liquid interface
- 3D spheroid
- Organ-on-chip

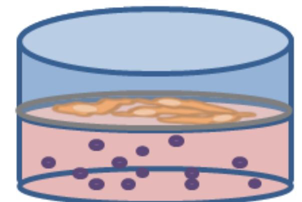
Submerged exposure (upright)



NP surface presentation



Inverted cell culture



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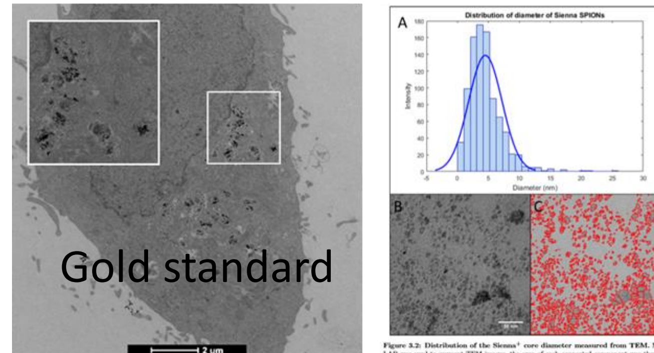
Biological experiments with nanoparticles

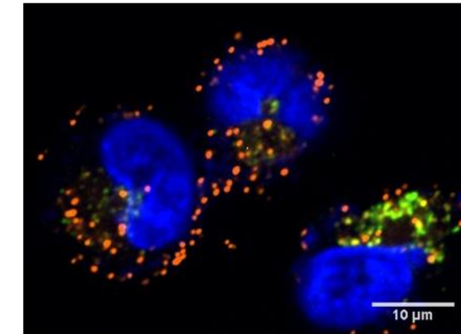
NANOGENTOOLS

- Characterization methods
- Dispersion
- Dosing considerations
- Cell type and exposure configuration
- <u>Imaging method</u>
  - TEM is gold standard
  - Fluorescent is common in microscopy
  - Artefacts?
  - Reflectance is an alternative
  - Other methods also available with differing resolution and detection capabilities

Transmission Electron Microscopy

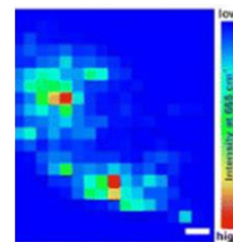


Fluorescence Microscopy



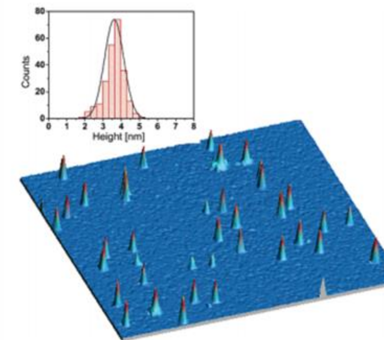
Drescher *et al* 2010

Raman

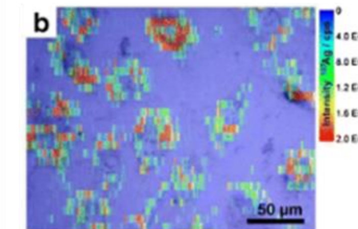


Drescher *et al* 2010

Atomic Force Microscopy



Laser-Ablation ICP-MS



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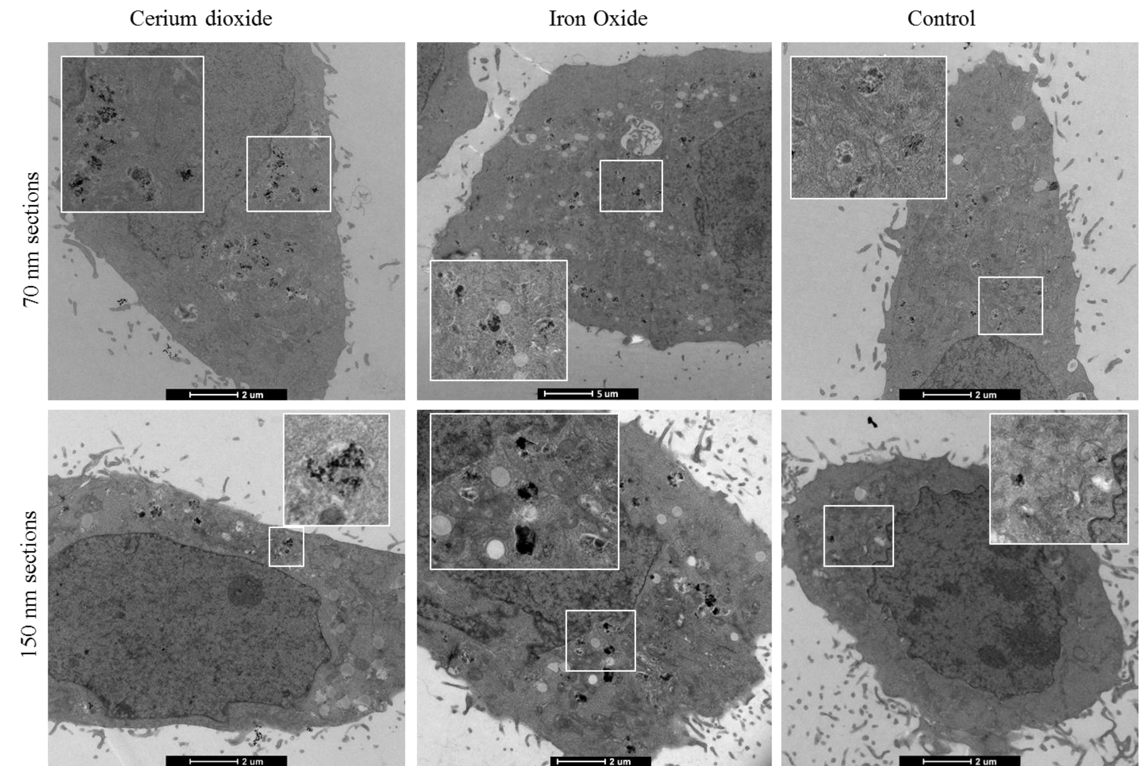
Microscopy methods available for intracellular NP imaging

Traditional TEM imaging

- TEM is the gold standard for imaging NPs
- Advantages
  - Ultrastructure contrast
  - Ultrahigh resolution
  - Individual NPs distinguished
- Limitations
  - Ultrathin sections required
  - Expertise for processing
  - Time consuming preparation
  - Limited cells can be imaged
  - Artefacts from processing?



Guggenheim et al 2016

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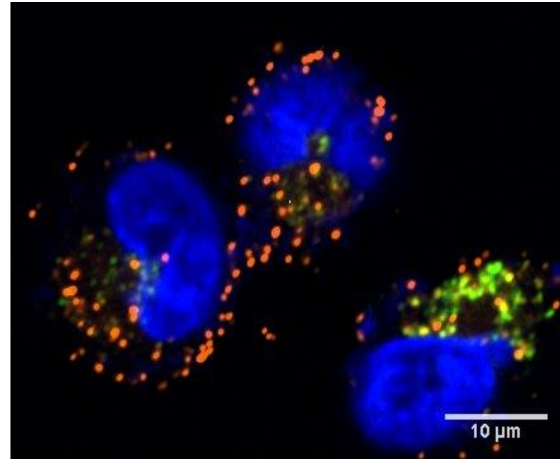
Light microscopy for NP detection in cells

NANOGENTOOLS

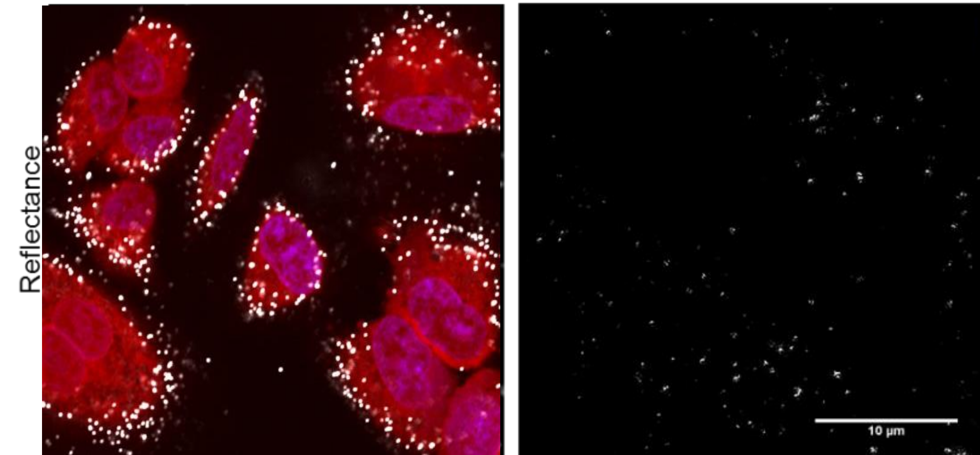
- Fluorescence
  - Widely available and used
  - Requires labels
  - Well established
  - Multiple labels
- Reflectance
  - Label free
  - Less well documented
  - High background signal

Fluorescence



Collection of light emitted from a fluorescent moiety attached at the NP surface following excitation

Reflectance



Collection of light reflected back from optically dense NP – relies on changes in refractive index between NP and the surroundings

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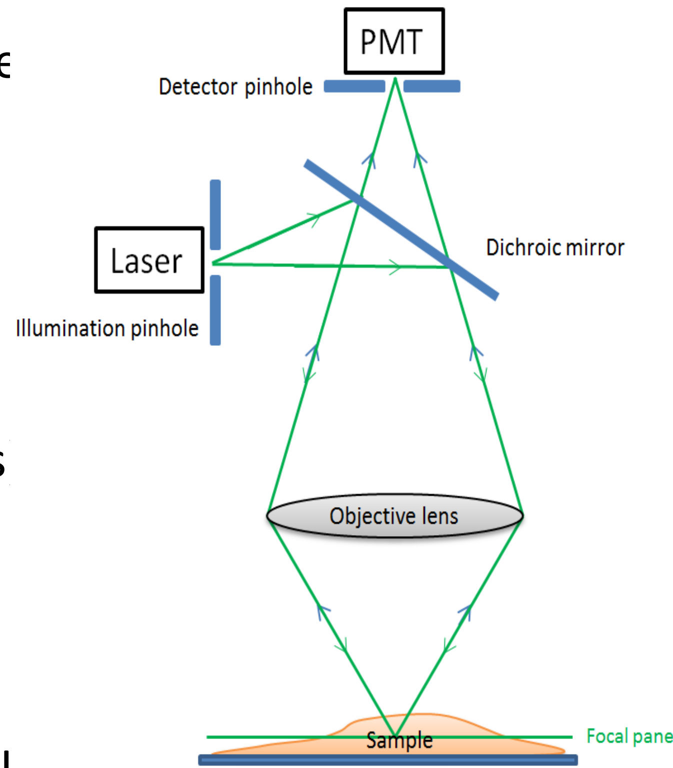
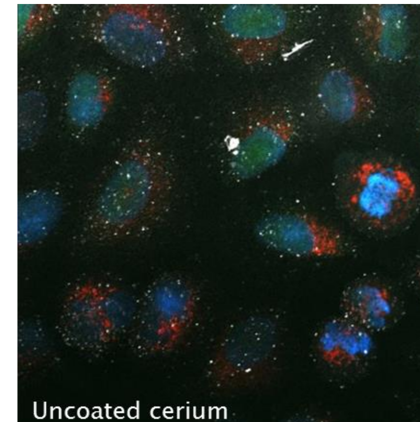
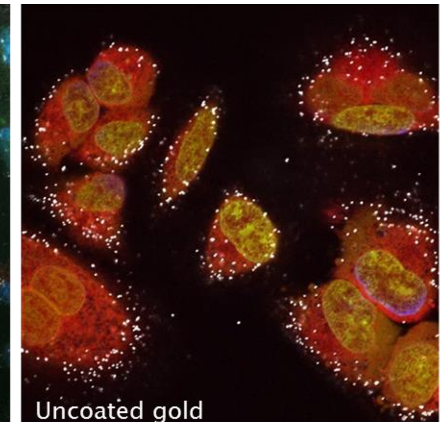
Reflectance Confocal

- Contrast enhancement in reflectance imaging using confocal microscopy
- Performed on the same instrumentation as for fluorescent imaging – widely available
- Can be combined with fluorescent staining (i.e. cell, nucleus, organelles
- Allows 3D imaging of live and fixed cells
- Quick and little sample prep
- Diffraction limited – ~300 nm laterall and ~700 nm axially





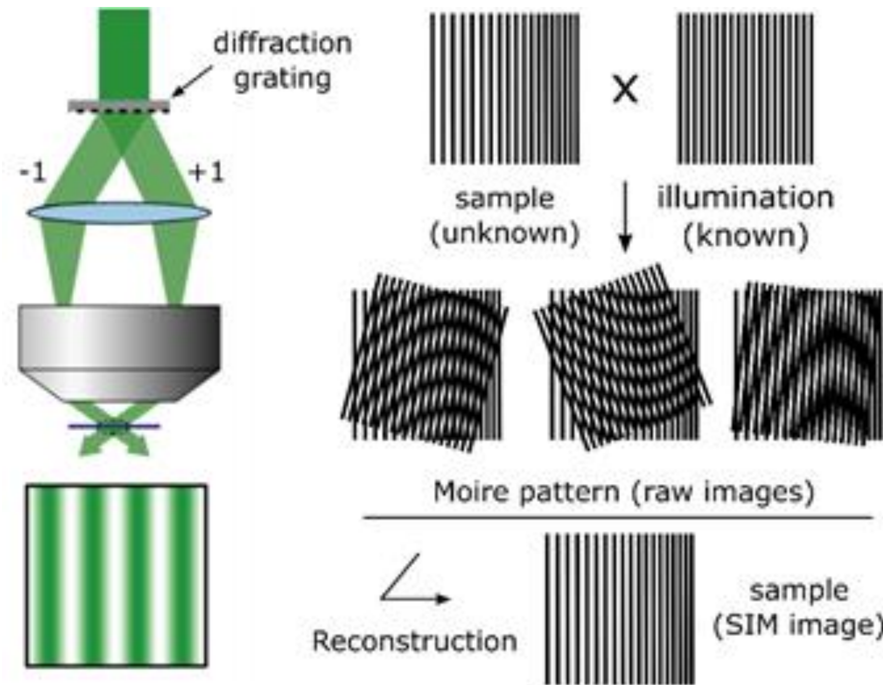


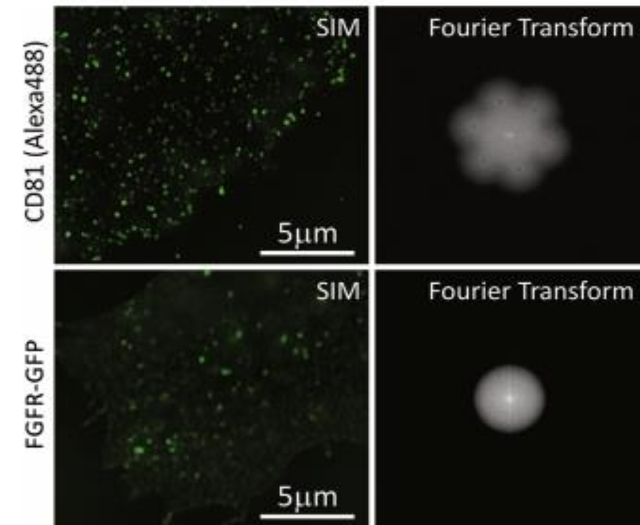
Guggenheim et al 2016

Super-resolution imaging

NANOGENTOOLS

- Allow acquisition of images with resolution greater than the diffraction limit
- Widely available techniques for fluorescent imaging – STORM/PALM, STED, ExpMic
- Very limited examples of super-resolution reflectance
- Structured illumination (SIM) allows the acquisition of 2-fold greater resolution
- Uses a grating to project excitation light at several angles and phase
- Reconstructed in Fourier space to give the FT image with 'superresolution' data
- Inverse FFT gives the SR Image

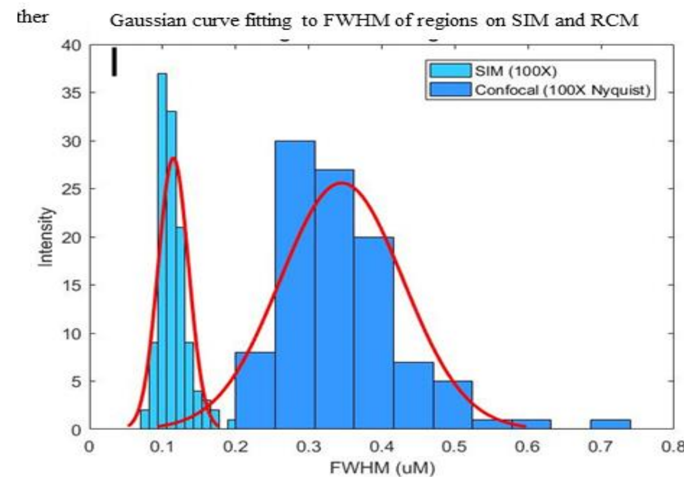


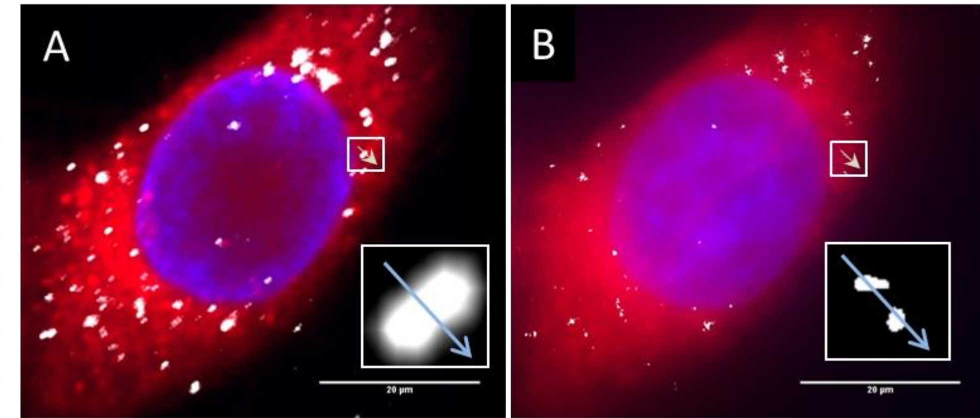


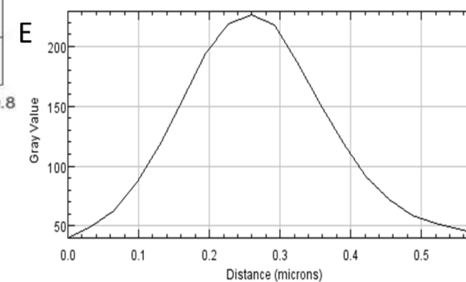
Andor: http://www.andor.com/learning-academy/super-resolution-imaging-structured-illumination-microscopy-application-note

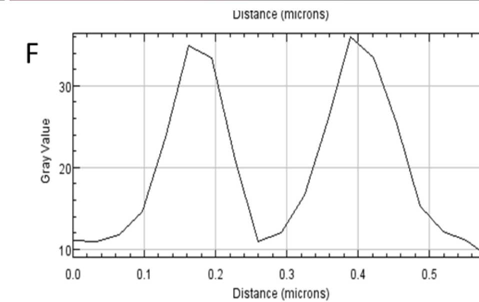
Super-resolution reflectance

- We have shown that SIM can be performed in reflectance mode by the addition of half mirror to the light path (N-SIM)
- No additional sample prep needed
- Two-fold increase in the maximal resolution achievable (115 nm)
- Allows separation of clusters previously unresolvable by RCM
- Advantageous for imaging of small structures (such as NP) that are smaller than the diffraction limit
- Minimise the uncertainty – ie in colocalization studies









Guggenheim et al 2016

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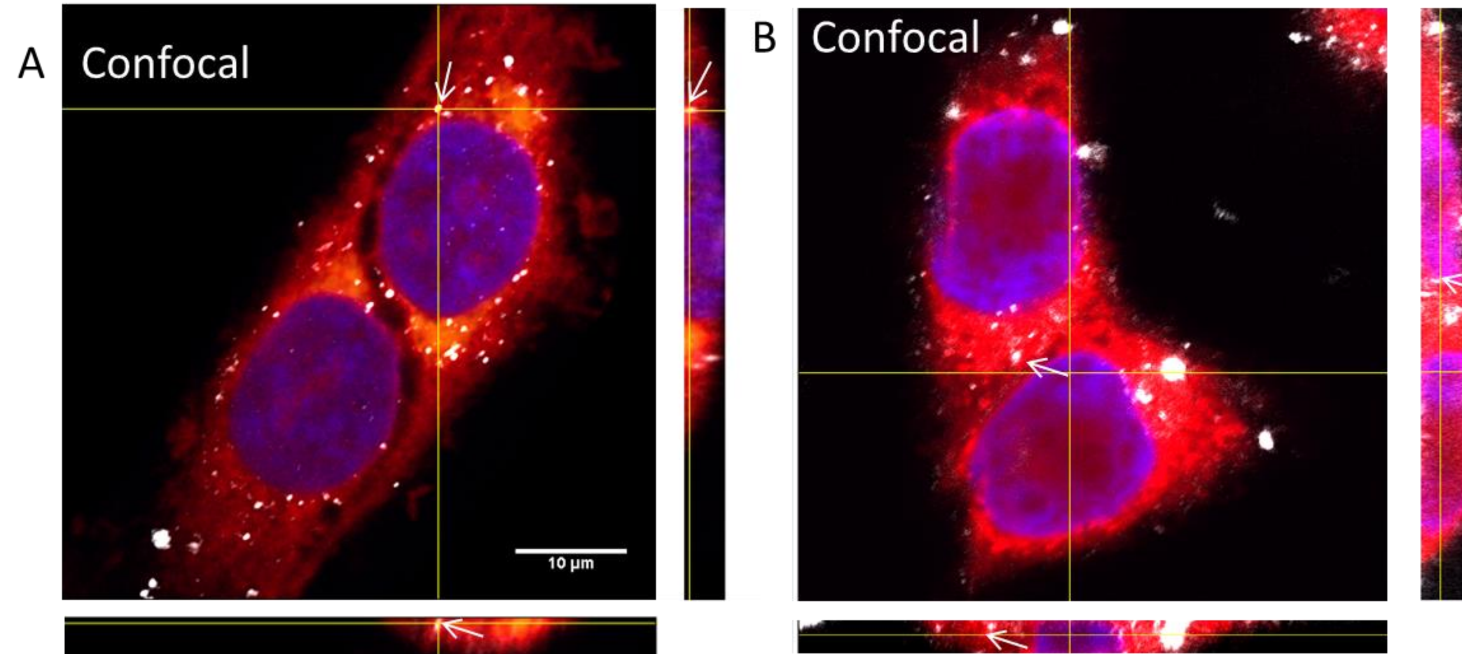
Quantification of NP uptake from images

- Quantification of this uptake is critical
  - Environmental safety studies
  - Biomedical studies where localization to within tumour cells is critical for toxic effects



Example of cancer cells (HeLa) treated with SPIONs (biomedical) and Cerium dioxide (added to fuels as catalytic converter)

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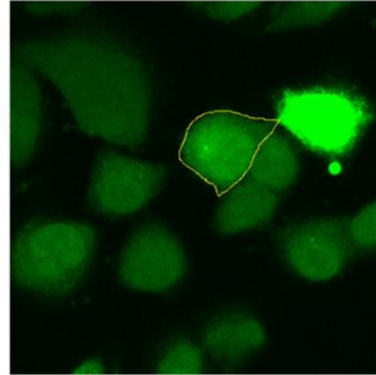
Quantification of NP uptake from images

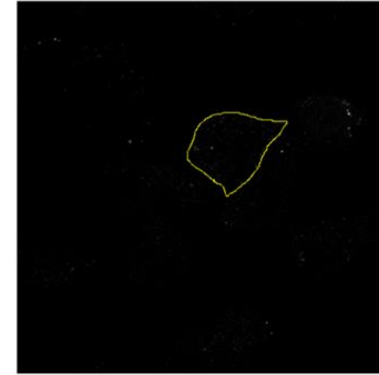
NANOGENTOOLS

- Quantification used to rely on manual analysis
- Manual delineation leads to user error and biased results
- Manual delineation is also time consuming

Draw an RIO using cytoplasmic stain



Transfer ROI to NP Channel and take mean intensity



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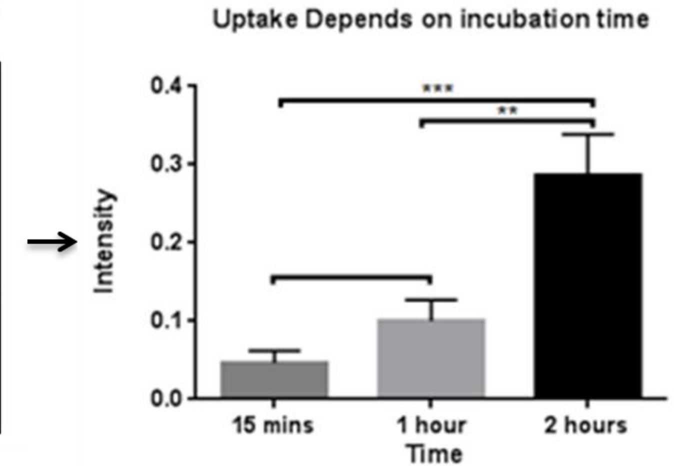

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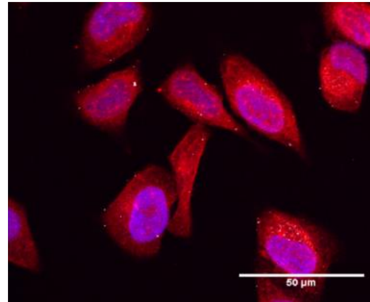
Quantification of NP uptake from images

- Automation of cell segmentation and NP segmentation
- Automation allows quick analysis of NP uptake under a variety of conditions
  - Same regions highlighted every single time
- Analysis can be applied to multiple data types
  - Confocal, SIM, TEM (where contrast is good)
  - Segmentation of cells, nuclei, NP signal, Fluorescent stains
  - 2D and 3D
- Less time consuming, therefore throughput and reliability is increased
  - 1000s cells analysed in a few mins

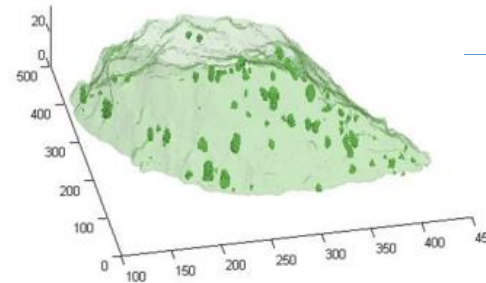
1. Aqcuire image



2. Digital Matrix

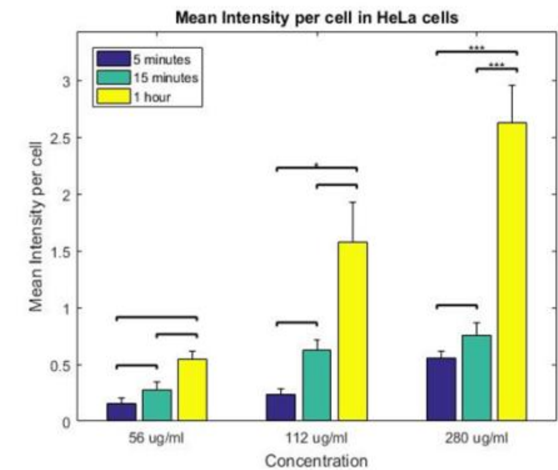
```
08 02 22 97 38 15 00 40 00 75 04 05 07 78 52 12 50 77 91 08
49 49 99 40 17 81 18 57 60 87 17 40 98 43 69 48 04 56 62 00
81 49 31 73 55 79 14 29 93 71 40 67 53 88 30 03 49 13 36 65
52 70 95 23 04 60 11 42 69 24 68 56 01 32 56 71 37 02 36 91
22 31 16 71 51 67 63 89 41 92 36 54 22 40 40 28 66 33 13 80
24 47 32 60 99 03 45 02 44 75 33 53 78 36 84 20 35 17 12 50
32 98 81 28 64 23 67 10 26 38 40 67 59 54 70 66 18 38 64 70
67 26 20 68 02 62 12 20 95 63 94 39 63 08 40 91 66 49 94 21
24 55 58 05 66 73 99 26 97 17 78 78 96 83 14 88 34 89 63 72
21 36 23 09 75 00 76 44 20 45 35 14 00 61 33 97 34 31 33 95
78 17 53 28 22 75 31 67 15 94 03 80 04 62 16 14 09 53 56 92
16 39 05 42 96 35 31 47 55 58 88 24 00 17 54 24 36 29 85 57
86 56 00 48 35 71 89 07 05 44 44 37 44 60 21 58 51 54 17 58
19 80 81 68 05 94 47 69 28 73 92 13 86 52 17 77 04 89 55 40
04 52 08 83 97 35 99 16 07 97 57 32 16 26 26 79 33 27 98 66
88 36 68 87 57 62 20 72 03 46 33 67 46 55 12 32 63 93 53 69
04 42 16 73 38 25 39 11 24 94 72 18 08 46 29 32 40 62 76 36
20 69 36 41 72 30 23 88 34 62 99 69 82 67 59 85 74 04 36 16
20 73 35 29 78 31 90 01 74 31 49 71 48 86 81 16 23 57 05 54
01 70 54 71 83 51 54 69 16 92 33 48 61 43 52 01 89 19 67 48
```

3. Segment all objects of interest



2D and 3D

4. Measure parameters and display



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Application of techniques to different NP studies

Types of cell studies

- Cellular uptake potential
- Cell toxicity
- SiRNA studies to determine route
- Colocalisation studies to determine fate
- Uptake in more physiologically relevant models
- Single Cell ICP-MS

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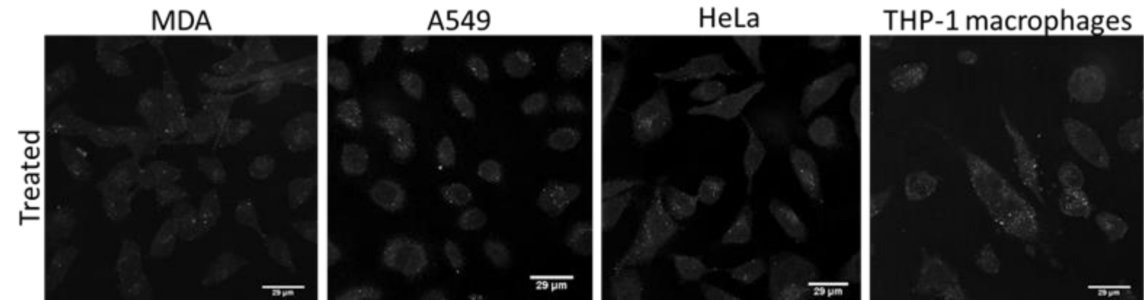
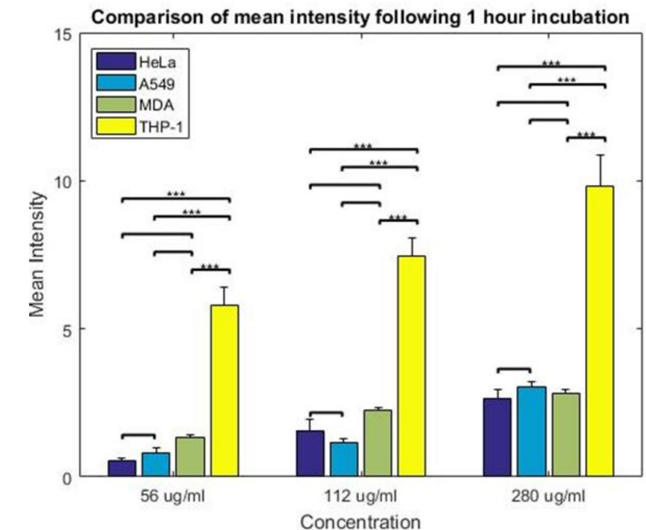
Uptake of NPs in cancer cells

- Different cell types exposed to NP to determine uptake patterns
  - Applied to a host of different particles: Cerium, Gold, Titania, Zinc, Iron oxide, Polystyrene
- Imaged using reflectance confocal
- SPIONs show dose dependant increase in uptake
- Different cells show different SPION uptake levels
- Measured automatically using MATLAB: over 1500 cells analysed, increasing reliability and throughput of studies



Cancer cells were determined to internalize significantly less NP into cells when compared to macrophage cells



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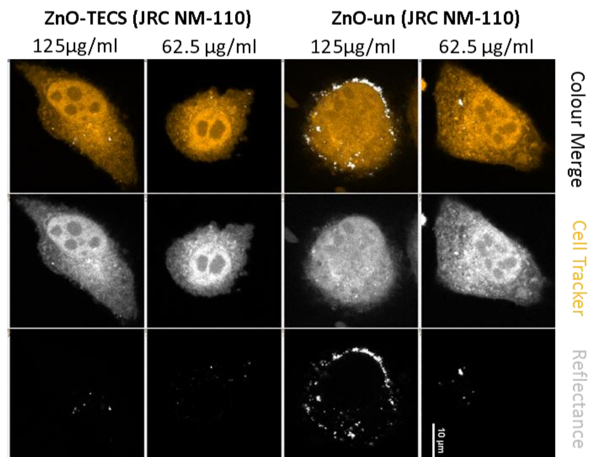
NP screen for celluar uptake

- Similar studies were applied to NPs used in NanoMILE project to perform a screen of an NP library to determine uptake using reflectance confocal imaging



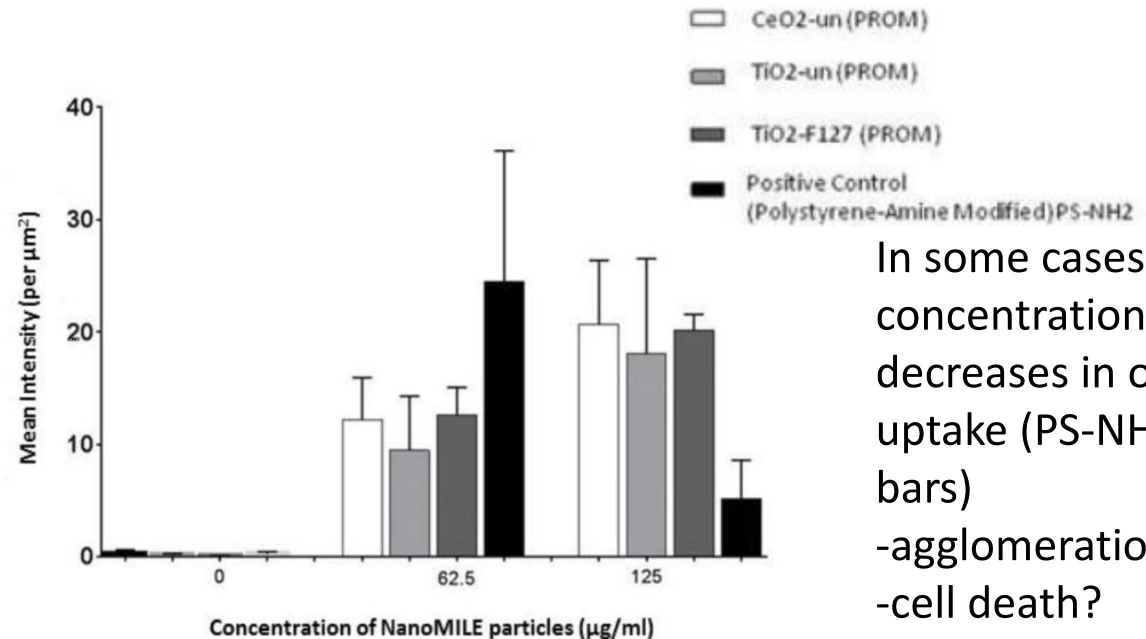


Figure 33_UoB: Graph showing dose response in mean intensity detected after treating A549 cells with selected phase I NanoMILE MNMs at 62.5 µg/ml, and 125 µg/ml. Images are treated with background subtraction to reduce control values and accurately reflect mean intensities within MNM treated cells. The cell area from which mean intensity values are derived is defined by 'regions of interest' drawn around Cell Tracker Orange stained cells. 30 cells per replicate are analysed, *n*=3.

In some cases increases in concentration lead to decreases in observed uptake (PS-NH_2 NPs – black bars)
-agglomeration?
-cell death?

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Library screen for NP uptake – PS-NH_2 NPs

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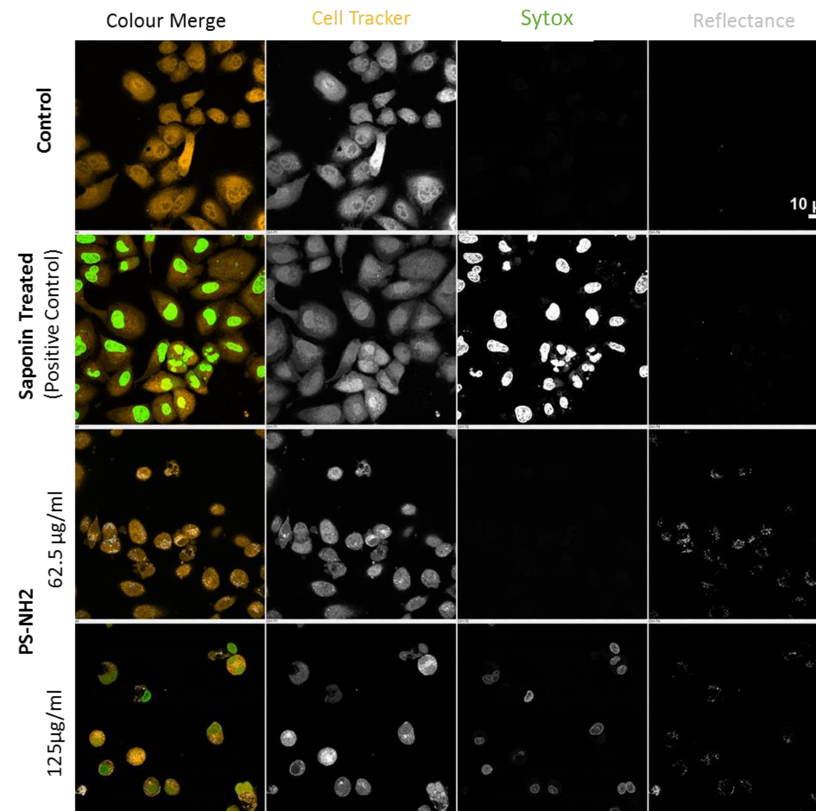
- Similar studies were applied to NPs used in NanoMILE project to perform a library screen to determine uptake using reflectance confocal imaging
- This was combined with SYTOX fluorescent staining for toxicity
  - SYTOX stains nuclei green when cell membranes are compromised
- Toxicity manifests as a fewer number of small more rounded cells
- Determine the dose dependant increase in toxicity of PS-NH_2 NPs



PS-NH_2 NPs cause increased cellular toxicity with increased exposure concentration, leading to increased cell death and apparent decrease in the observed NP uptake when measured

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Toxicity of NP based on cellular segmentation

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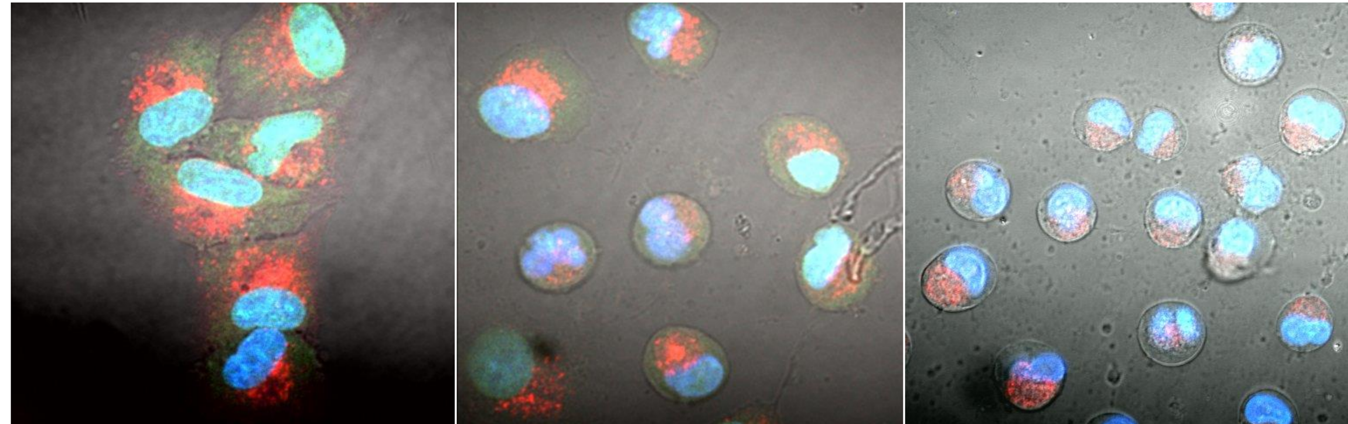
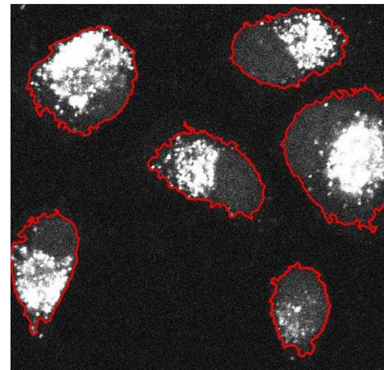
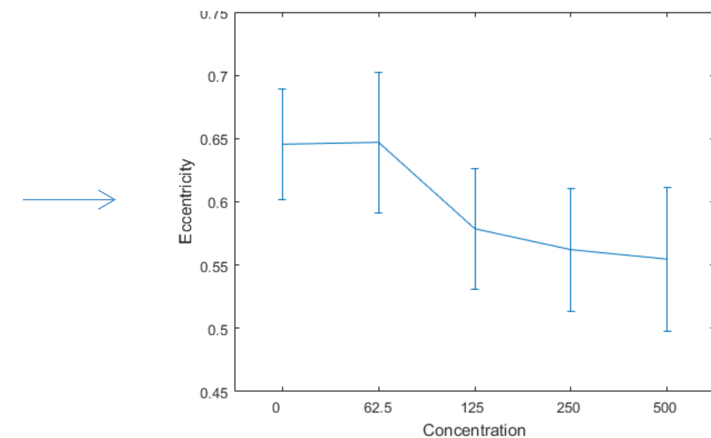
- Cell size and rounding are indicators of toxicity
- Toxicity can therefore be assessed based on changes to cell size and shape
- Automated analysis of cell shape gives a value of the eccentricity
  - Example: Ludox sillica
- Provides a high throughput mechanism of toxicity assessment for NPs (such as ludox in this case) (~60 seconds)







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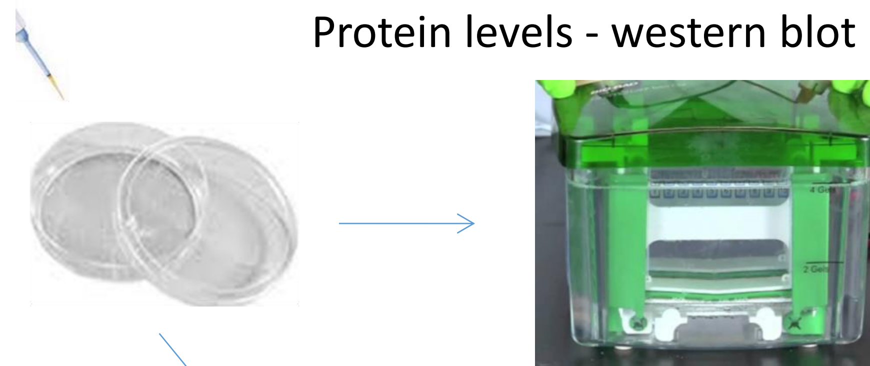
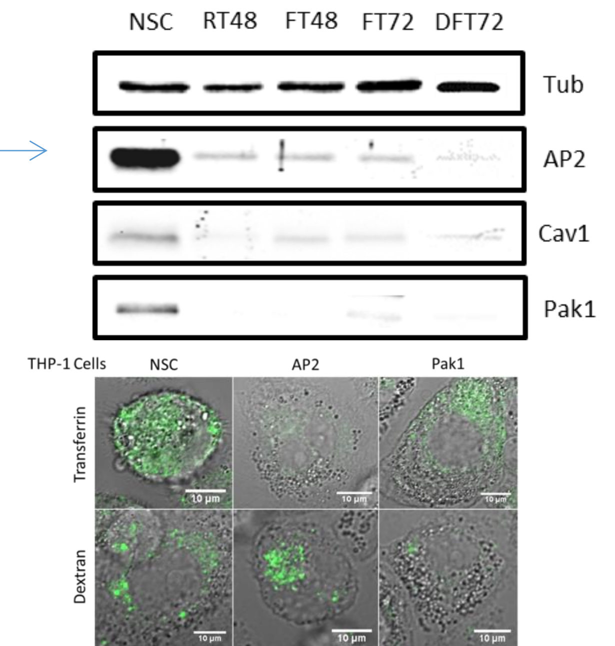
Mechanism of NP internalization

- <u>How do particles enter cells</u>?
- Pharmacological inhibitors
  - not that specific
  - often cause cellular toxicity
- siRNA
  - targets components of pathways specifically
  - More specific

Protein levels - western blot





Confocal imaging: visualize uptake

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Mechanism of NP internalization

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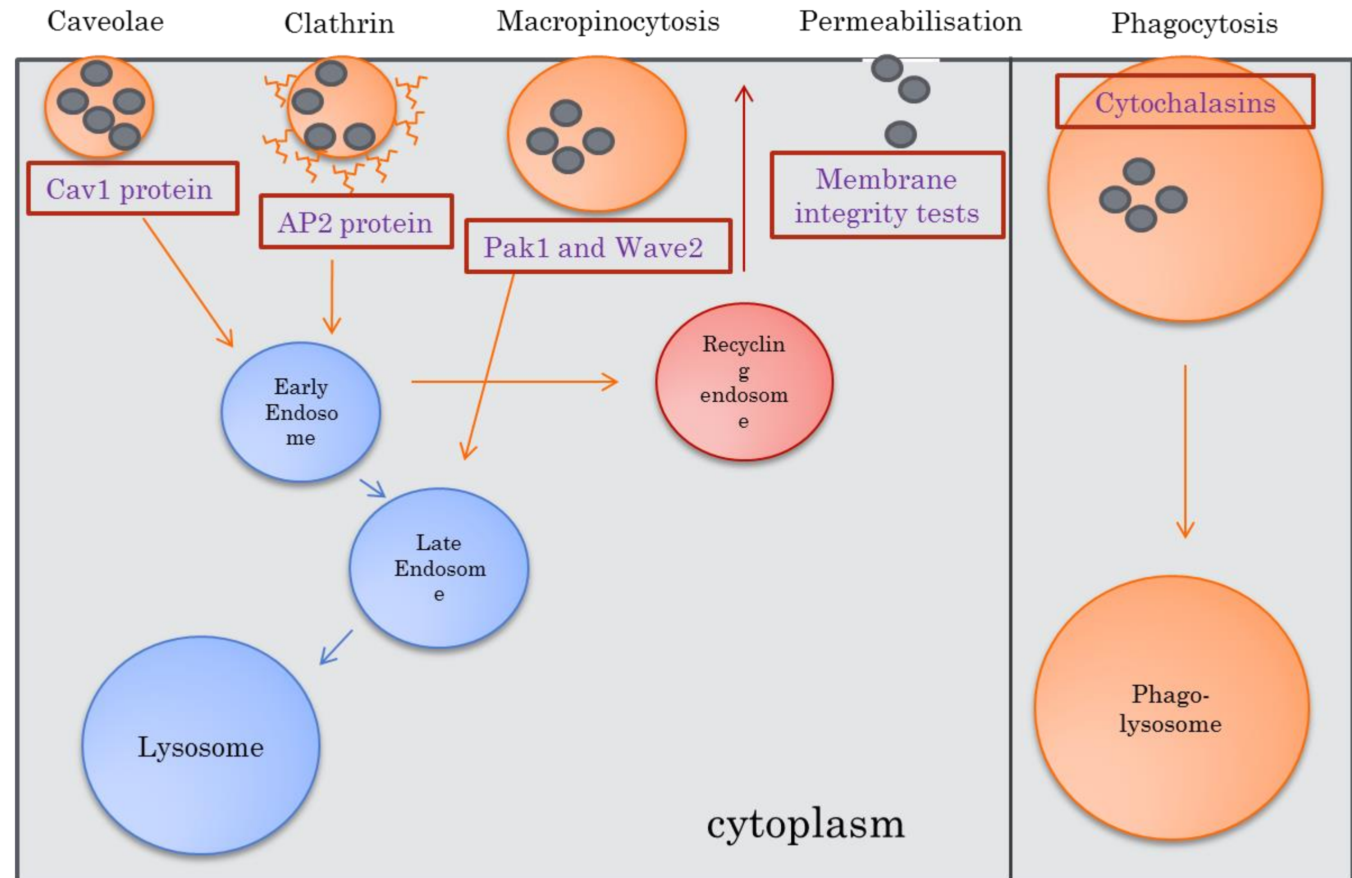
- How do particles enter cells?
- Use targetted siRNA
  - Cav1 inhibit cavealin mediated endocytosis
  - AP2 inhibits clathrin mediated endocytosis
  - Pak and Wave inhibit Macropinocytosis



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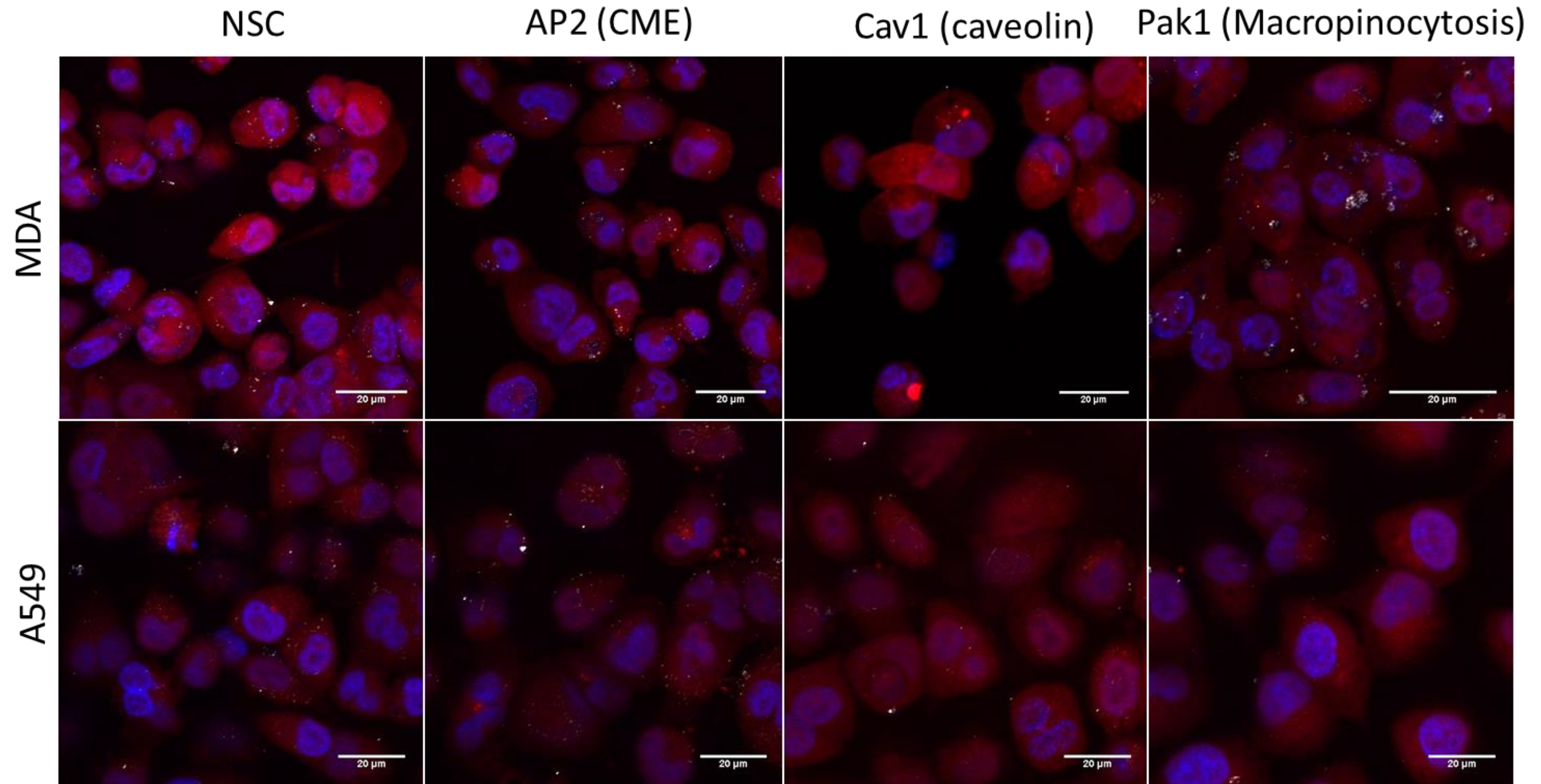
Mechanism of NP internalization

- How do particles enter cells?
- Treat different cell lines with different inhibitors and then visualize effect on NP internalization
- Imaged with confocal reflectance (NPs) – grey and fluorescence (Cell stain) red and blue
- Different uptake patterns seen in different cell types



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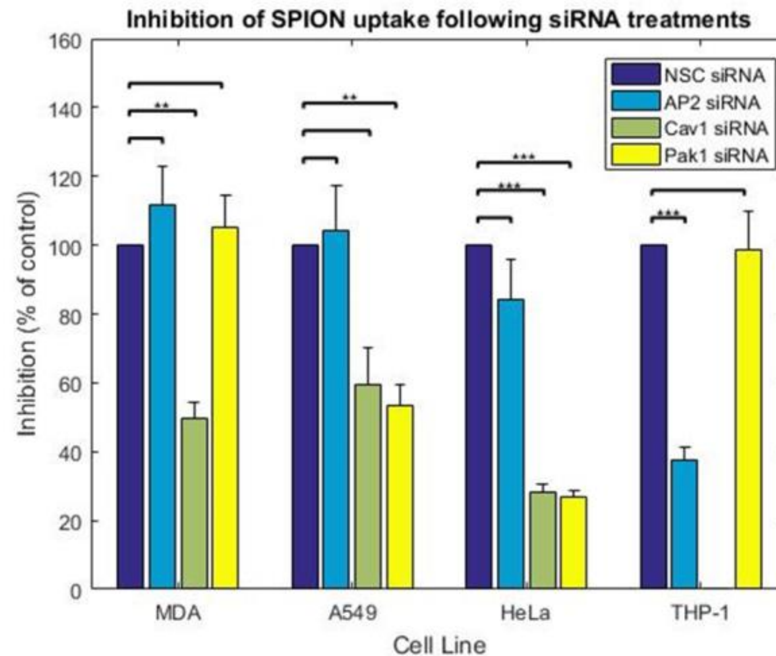
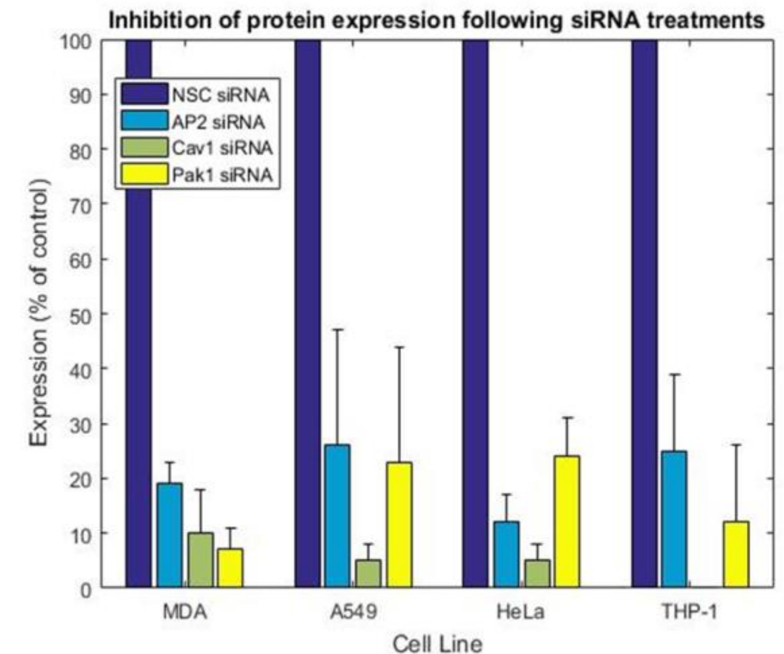
Mechanism of NP internalization

- How do particles enter cells?
  - In HeLa, A549, MDA cells the mechanisms implicated include cav-1 mediated (caveolae) and macropinocytosis
  - In Macrophages, mechanism appears to be dependant on AP2 and therefore indicates clathrin route
  - Differences may be due to properties: available receptors in cell types, corona constituents, active pathways



% Inhibition of uptake



% Inhibition of expression

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Subcellular localization of NP

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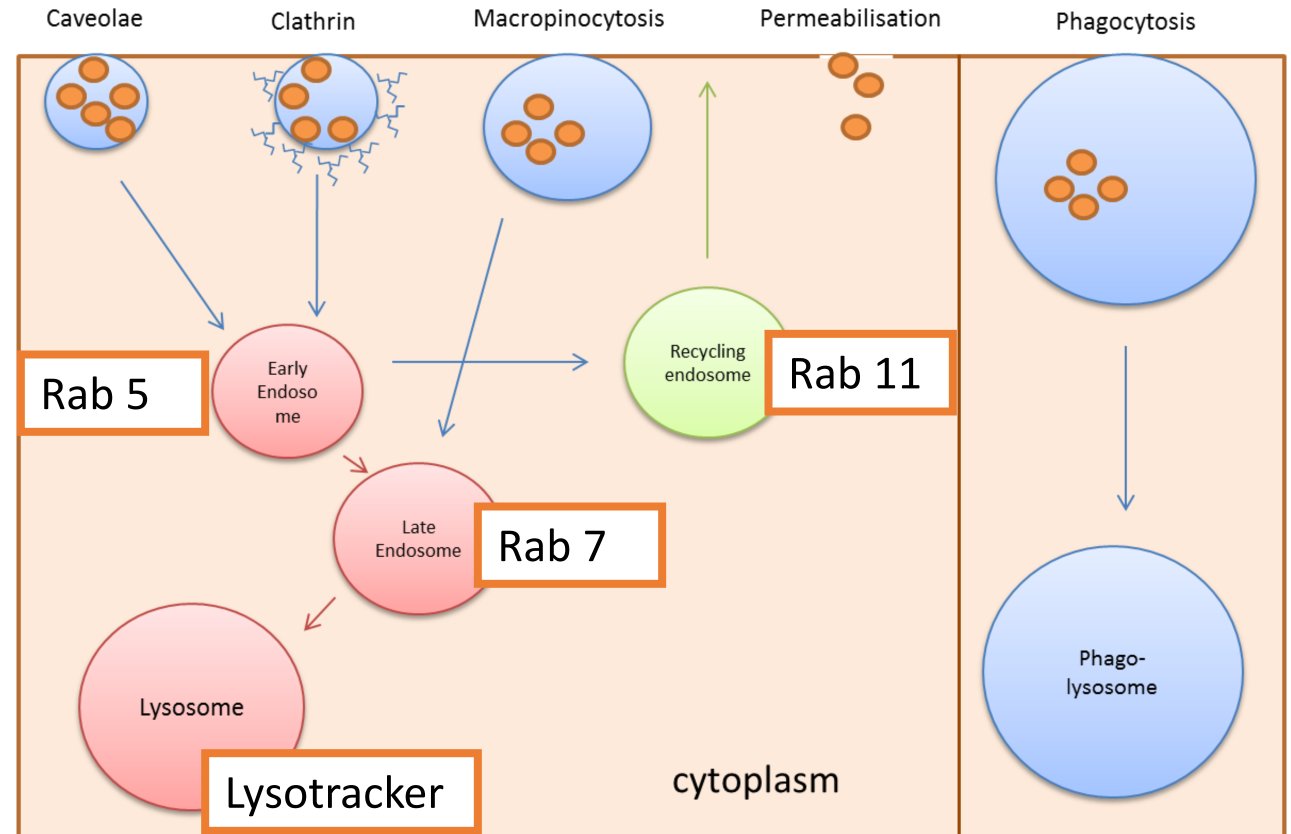
- Where do particles localize to within cells?
- Destination is important as it determines if/where a compound will be released and how NP will be metabolised by the cell (i.e. drug targeting)
- Colocalization studies with different endocytic marker proteins to identify routes taken following internalisation
- DNA transfection – fluorescently labelled proteins that mark compartments



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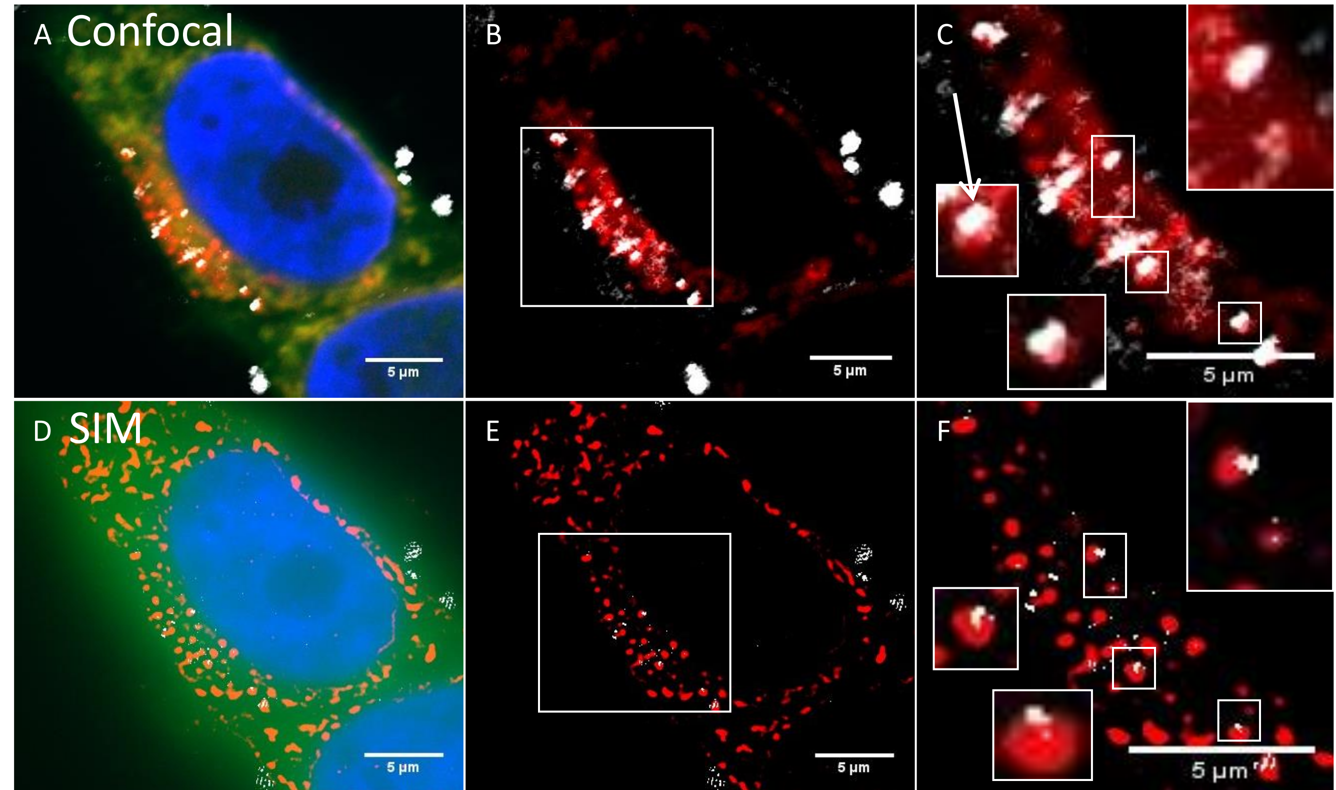
Subcellular localization of NP

- Where do particles localize to within cells? HeLa cell treated with SPIONs
- Confocal gives an indication of NP localization to a particular organelle
- SIM can give increased resolution and increased certainty in colocalization studies
- Aid nanocarrier design
- Inform on the potential bioaccumulation effects and potential success as drug delivery agents



Cells exposed to NP for 1 hour localise to lysosome: Red signal is lysosome and grey is NP

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Quantification of colocalization - endosomes

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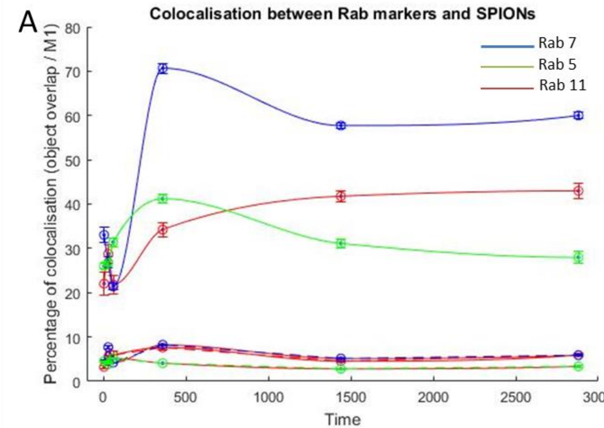
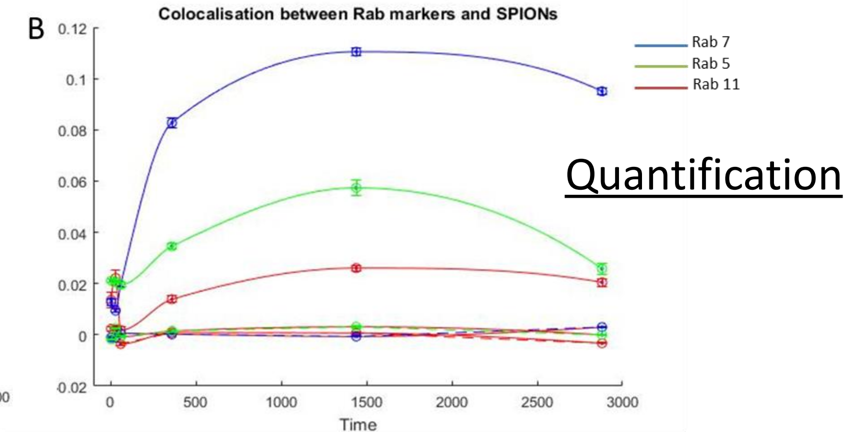
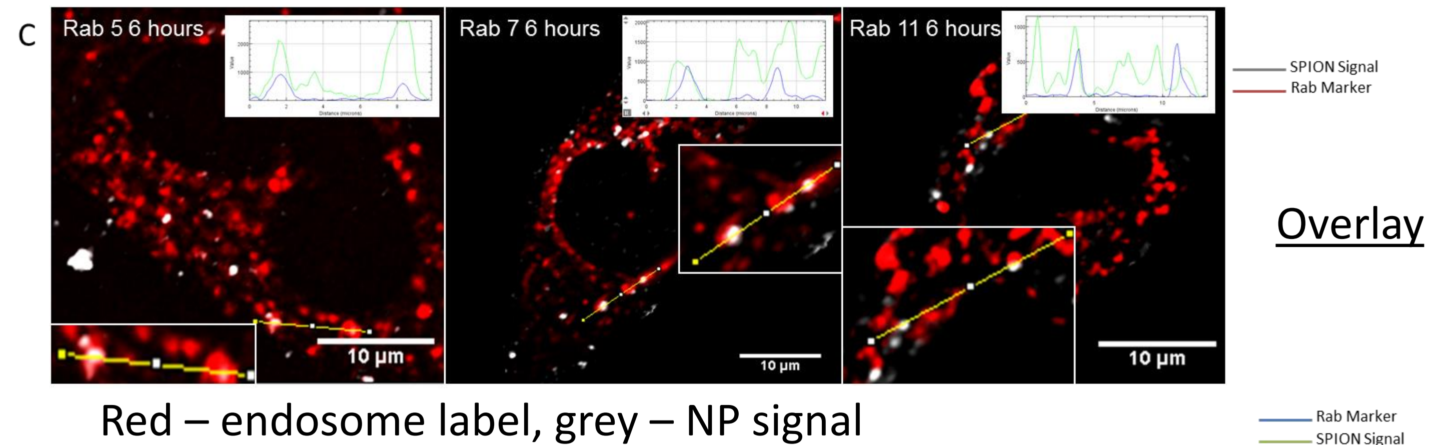
- Quantification used to rely on colour merges and overlays – although these can be useful they are not always reliable
- Automated analysis allows quantification in terms of Pearsons or Manders correlation coefficient giving colocalization a value
- SPIONs seen to colocalize with Rab 5 at the earlier time points
- Increases in colocalization with Rab 7 and 11 over time





Quantification



Overlay

Red – endosome label, grey – NP signal

Rab Marker
SPION Signal

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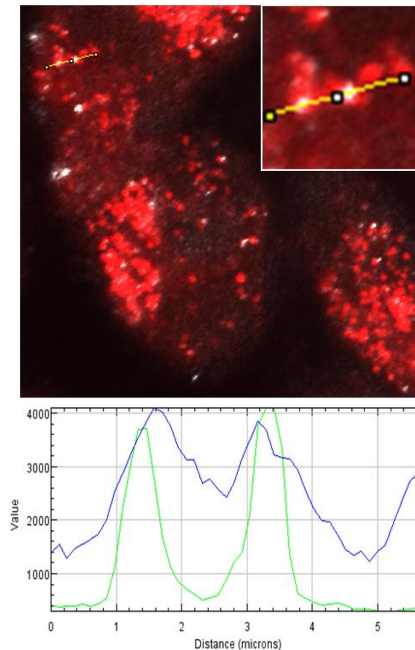
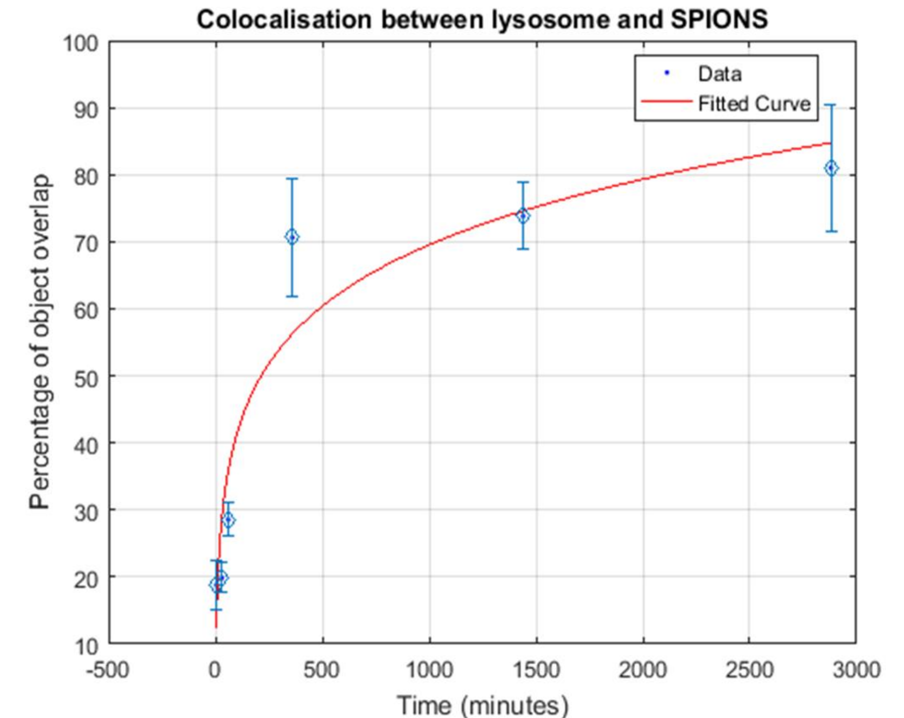
Quantification of colocalization - lysosomes

- Quantification used to rely on colour merges and overlays
- Automated analysis allows quantification in terms of Pearsons or Manders correlation coefficient giving colozalization a value
- SPIONs seen to increasingly colocalize with lysosome over time



Overlay



Quantification

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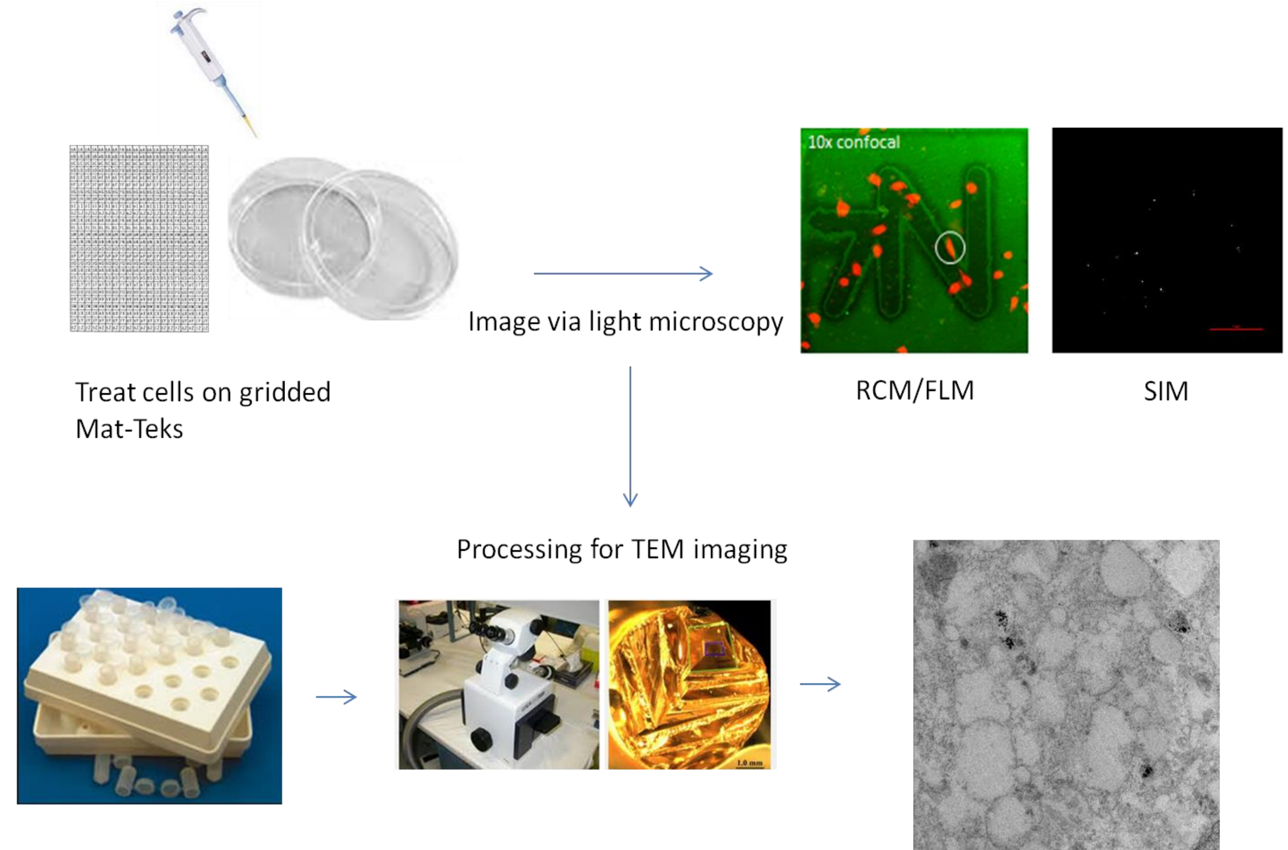
Correlative microscopy

- Correlating both light and electron microscopy offers advantages
- Visualize individual NP, fluorescent markers and reflectance signal
- Can confirm that nature of reflectance NP signal
- Procedure:
  - Plate cells onto dish with an alphanumeric grid
  - This grid is used as a reference to relocate cells of interest across modalities
  - Treat cells with NPs and fluorescent dyes
  - Image via LM, process for TEM, isolate grid
  - Image cells from grid square with TEM



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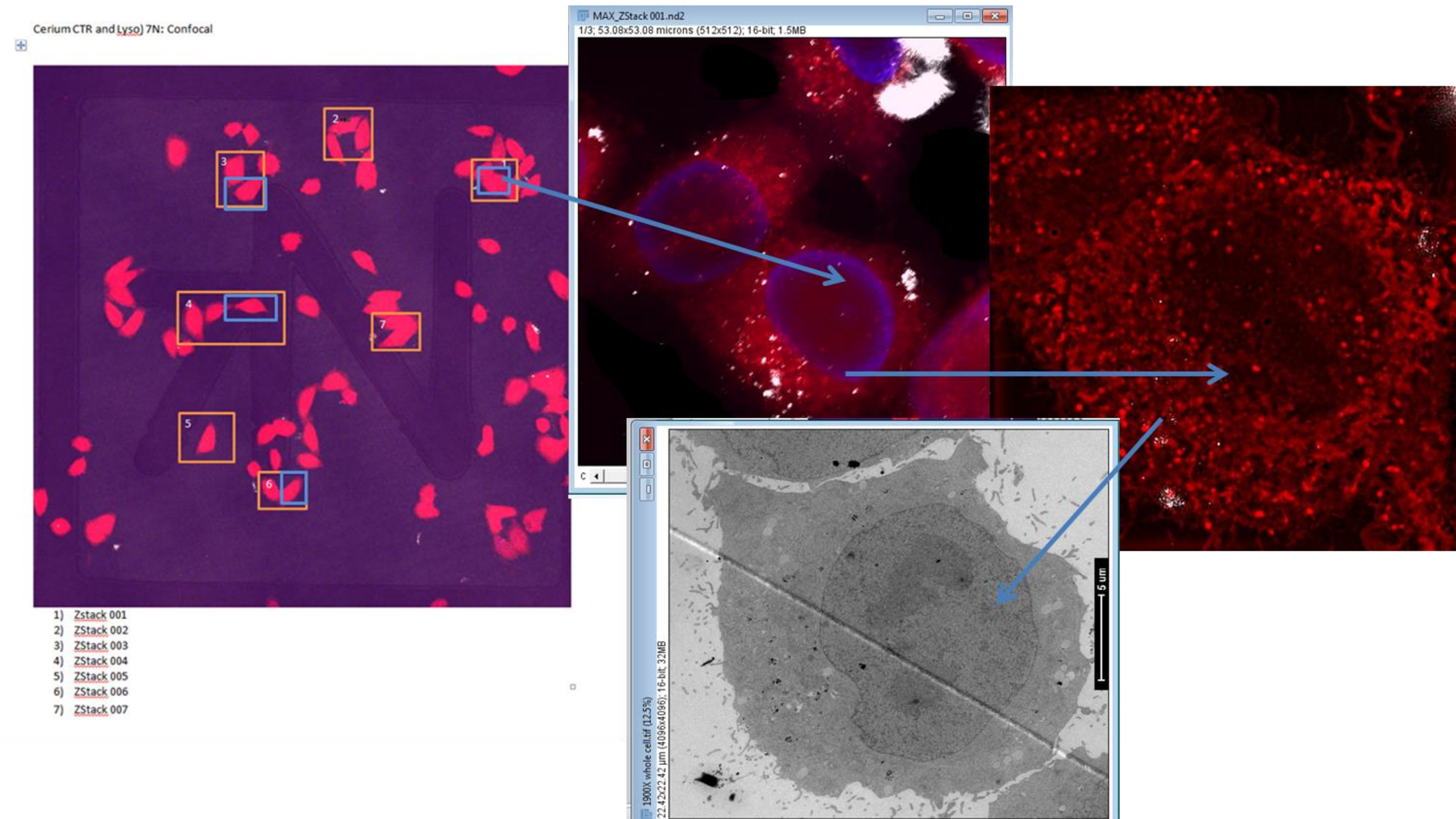
Correlative microscopy - optimization

- Correlating both light and electron microscopy offers advantages
- Challenges
  - Cell relocation across 3 modalities
    - Grid square enables relocation of cells
    - Maps created using cell positions on confocal (i.e. (7N)
  - TEM sample prep for LM / EM combo fixatives
  - Section thickness
  - Realignment challenges: semi-automated and automated



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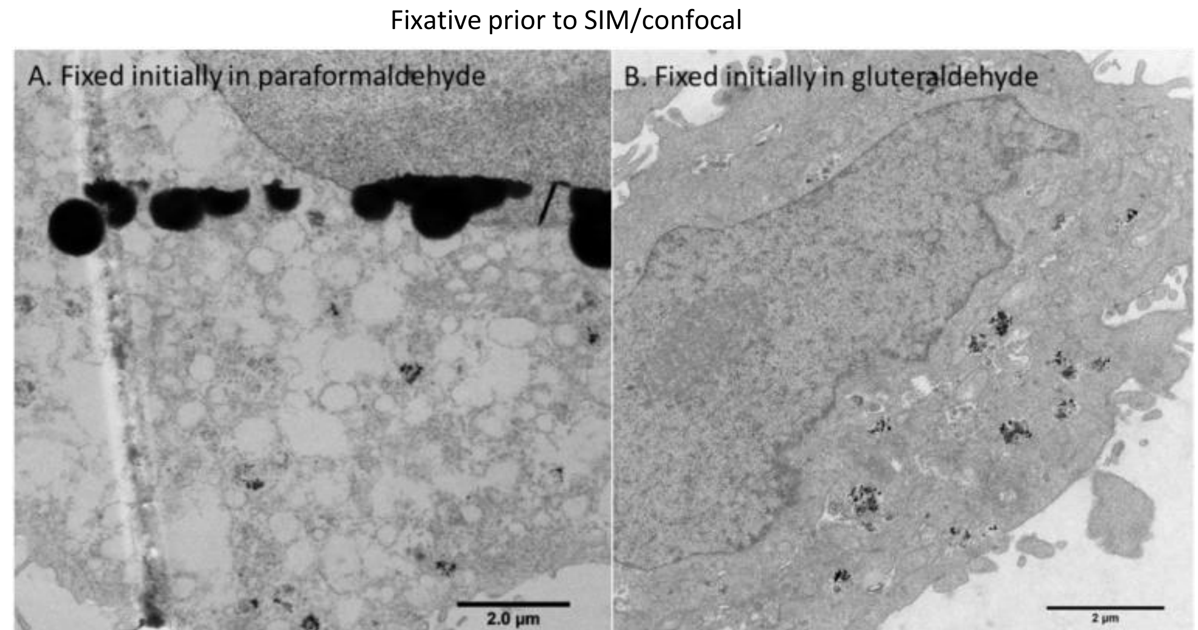
Correlative microscopy - optimization

- Correlating both light and electron microscopy offers advantages
- Challenges
  - Cell relocation across 3 modalities
  - <u>TEM sample prep for LM / EM fixatives</u>
    - PFA initial fix followed by GA/PFA leads to suboptimal preservation
    - GA/PFA initial fix followed by GA/PFA post fix led to optimal preservation
  - Section thickness
  - Realignment challenges: semi-automated and automated

Fixative prior to SIM/confocal



<u>Different fixatives led to different quality of TEM images</u>

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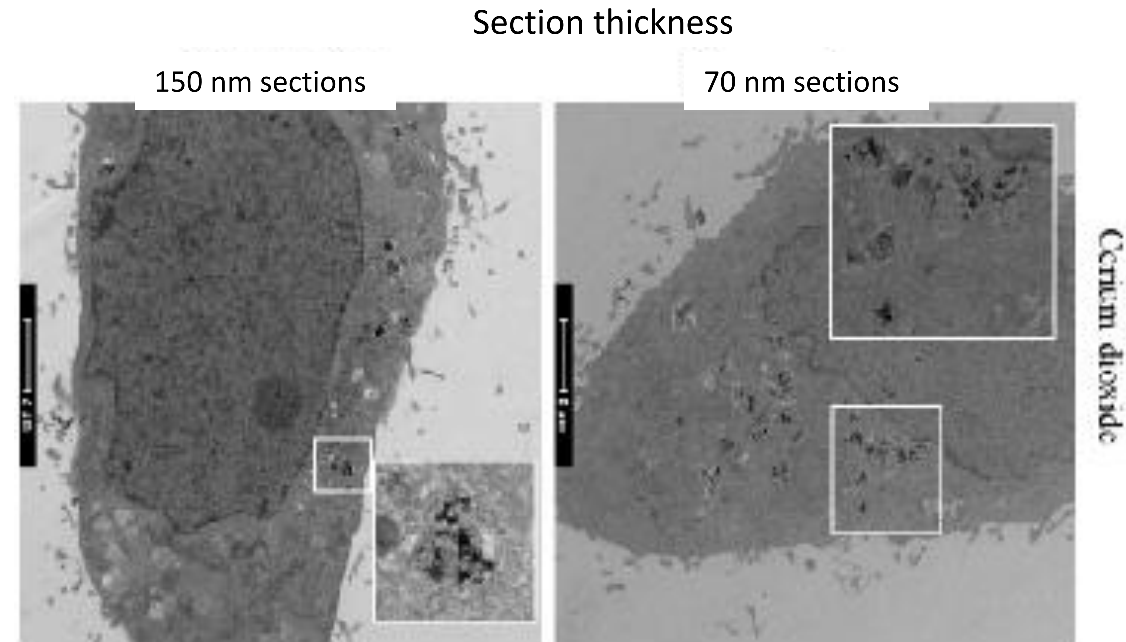
Correlative microscopy - optimization

NANOGENTOOLS

- Correlating both light and electron microscopy offers advantages
- Challenges
  - Cell relocation across 3 modalities
  - TEM sample prep for LM / EM fixatives
  - <u>Section thickness</u>
    - Thin sections differ greatly in Z-resolution to LM
    - Merging of thicker sections increased effective Z-resolution
  - Realignment challenges: semi-automated and automated

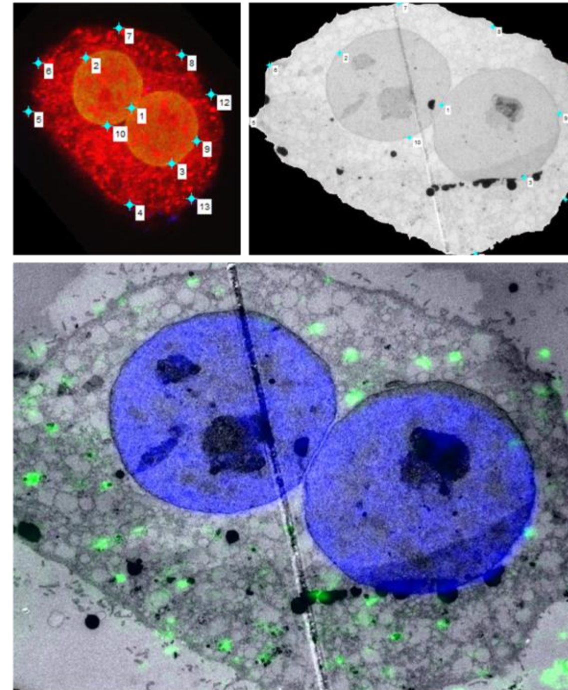


<u>Different section thickness aided realignment between modalities</u>
<u>Thicker sections better for correlative to LM</u>

Correlative microscopy - optimization

NANOGENTOOLS

- Correlating both light and electron microscopy offers advantages
- Challenges
  - Cell relocation across 3 modalities
  - TEM sample prep for LM / EM combo fixatives
  - Section thickness
  - <u>Realignment challenges: semi-automated and automated</u>



<u>Semi – automated</u>

User selects matching point pairs

Images are realigned using the computed tranformation

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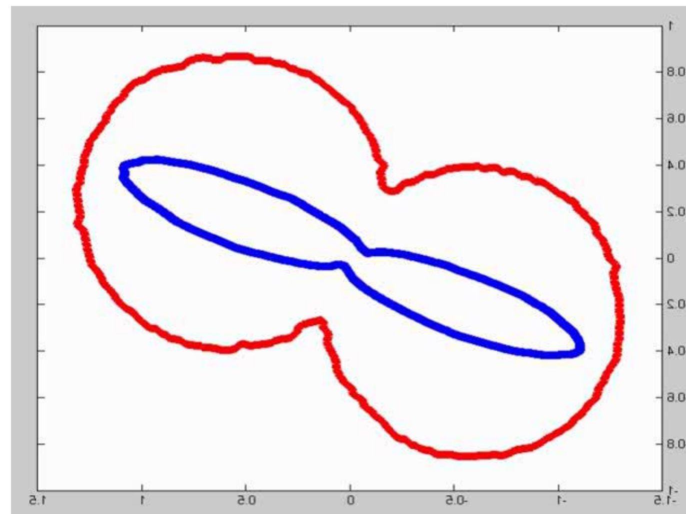
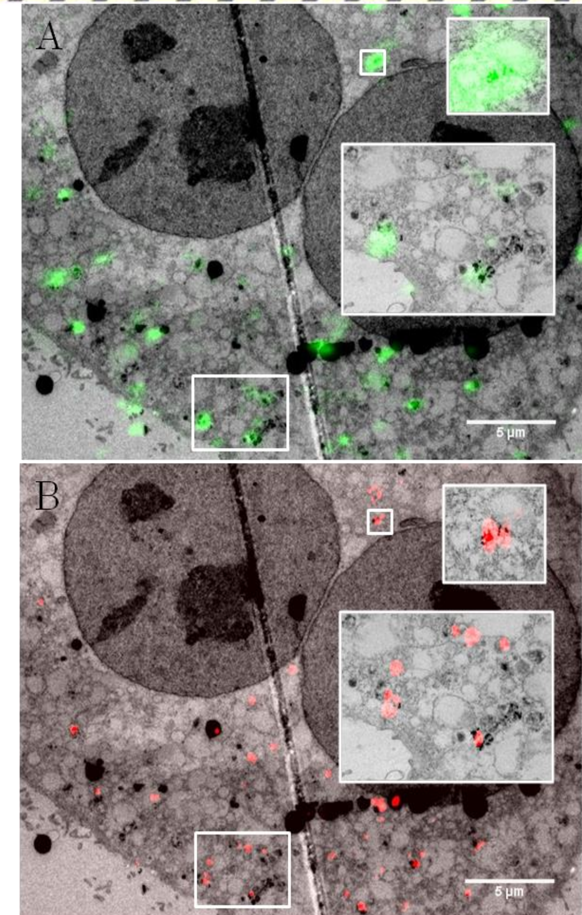
Correlative microscopy - optimization

NANOGENTOOLS

- Correlating both light and electron microscopy offers advantages
- Challenges
  - Cell relocation across 3 modalities
  - TEM sample prep for LM / EM combo fixatives
  - Section thickness
  - <u>Realignment challenges: semi-automated and automated</u>

<u>Automated: Coherant Point Drift Algorithm</u>





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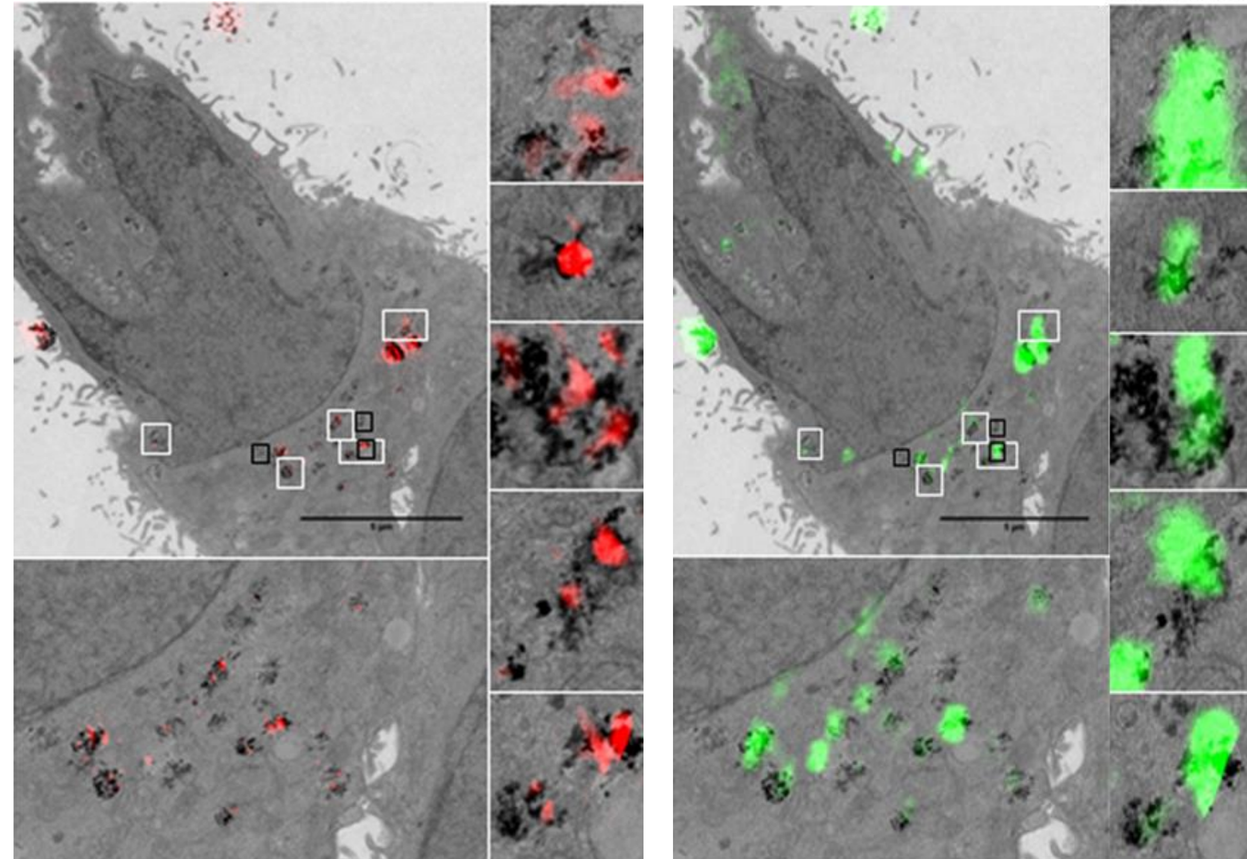
Correlative microscopy - Results

- NPs seen to localise to membrane bound regions with both R-SIM and RCM
- R-SIM provides increased resolution
- Although detection with reflectant methods is not as good as TEM, reflectance methods highlight a subset of particles



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Correlative microscopy - Results

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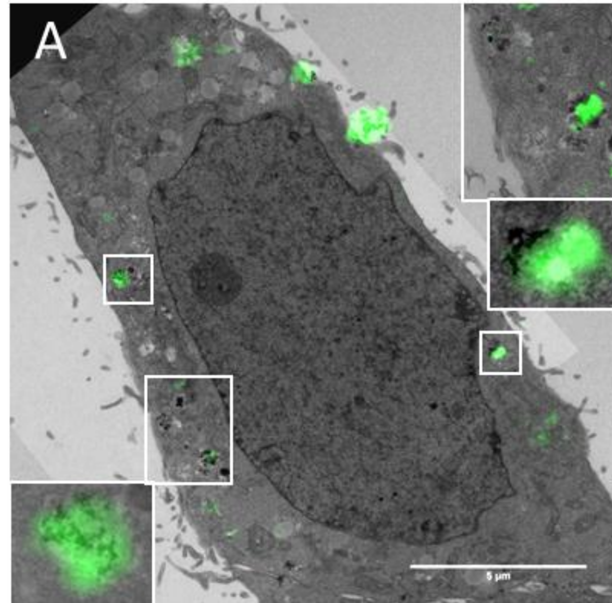
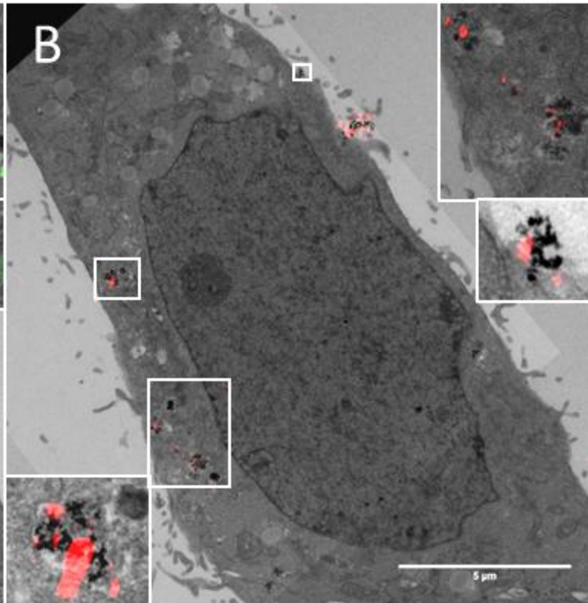
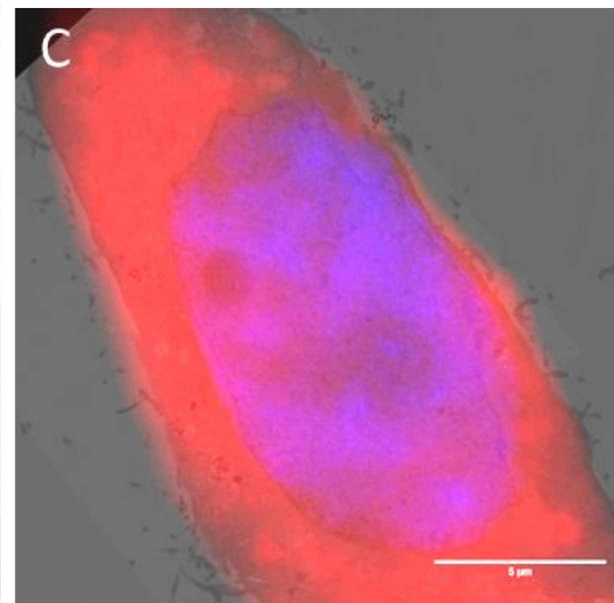
- Example including reflectance imaging of NPs and fluorescent cell staining of the nucleus, cell cytoplasm and lysosome



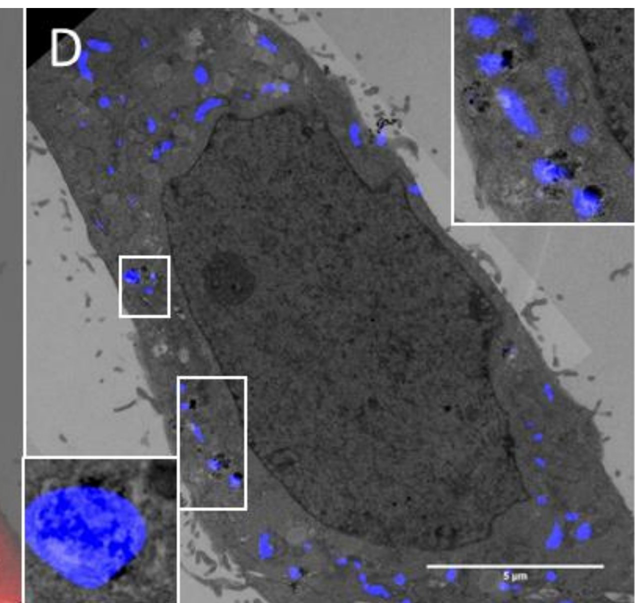
Reflectance Confocal (NPs – green)
TEM



Reflectance SIM (NPs – red)
TEM



Fluorescent DAPI (blue)
Fluorescent cytoplasm (red)
TEM



Fluorescent Lysosome (Blue)
TEM

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More representative systems: spheroids

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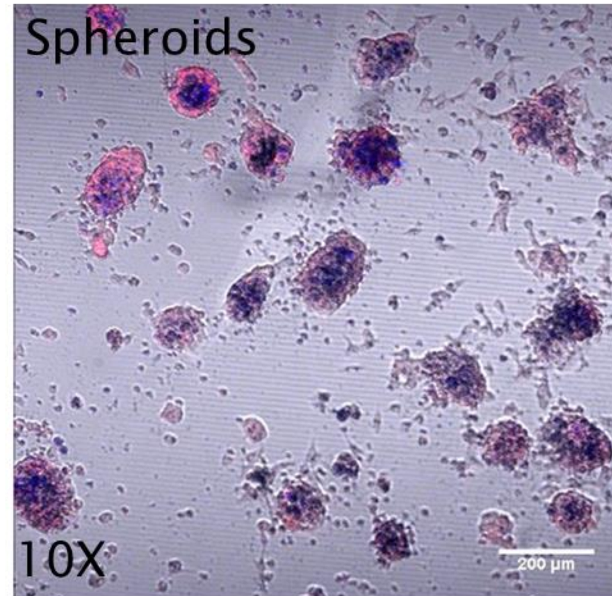
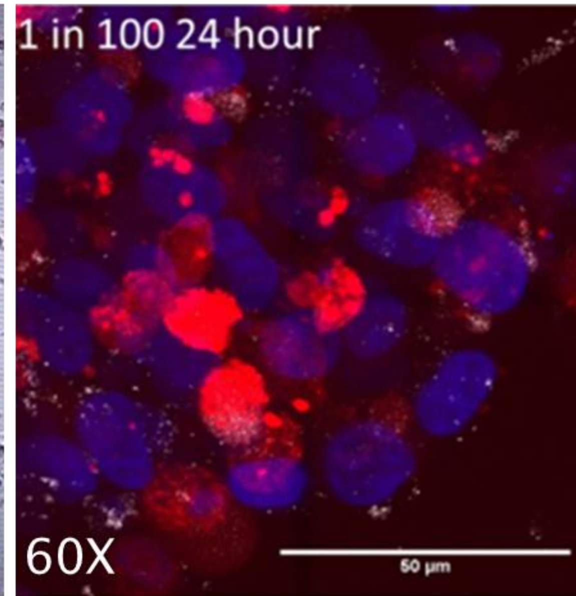
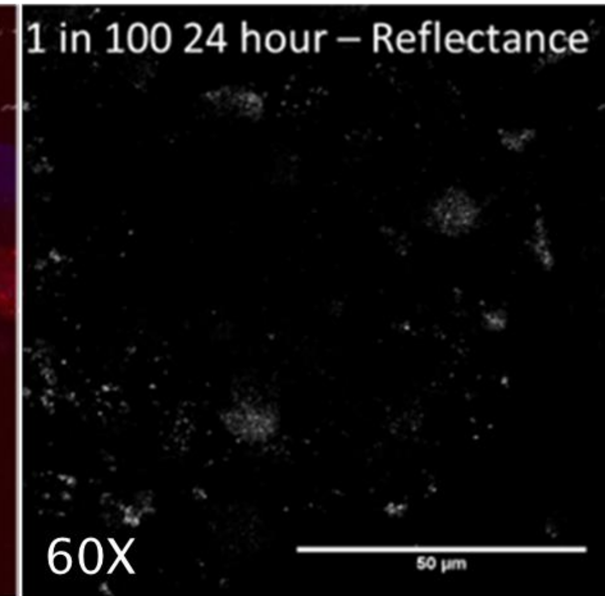
- Spheroids are 3D cellular structures that can be treated in similar ways to monolayers
- Are more representative of physiological conditions
- Can be engineered to have multiple cell types
- Extend to correlative microscopy and potentially *in vivo* studies



Spheroids grown on nanoimprinted surface



Fluorescent staining of spheroids



Nanoparticle uptake intro spheroids

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Single Cell ICP-MS

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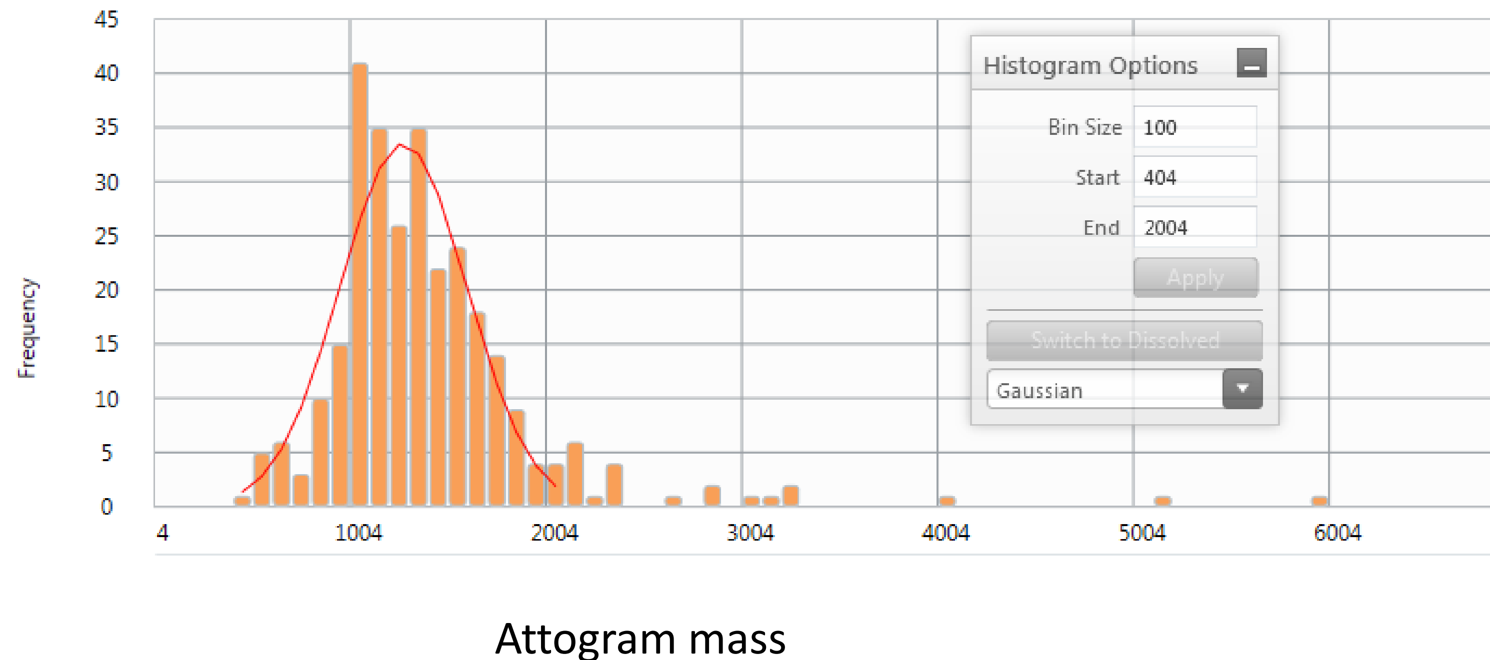
- New technique that allows introduction of an entire cell into a ICP-MS
- Birmingham owns one of the first SC-ICP-MS
- Samples of Algae cells following AuNP uptake
- Mass is determined in attograms (ag)
- Currently working on single cell particle NP uptake analysis in cancer cells and spheroids and correlating this with identification of the corona constituents



Attogram mass

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Conclusions

- It is crucial to understand the effects of NP exposure on cells to translate the potential risk posed to human health and the environment and maximize their beneficial use in biomedicine
- Although TEM is the gold standard for analysis, multiple imaging methods can be performed to evaluate single cells alone or in combination, each offering different advantages
- Light microscopy allows subcellular resolution and localization information regarding NP in 2D and 3D cultures including
  - Uptake
  - Trafficking
  - Fate
  - Toxicity
- New emerging techniques such as single cell ICP-MS will enable high throughput characterization of NP uptake

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Acknowledgements

The University of Birmingham

- School of Biosciences and Earth and Environmental Sciences
  - Prof. Iseult Lynch
  - Prof. Eva Valsami-Jones
  - NanoMile FP7 – (grant agreement No 310451)
  - H2020 ACENano (grant agreement No 60195)
  - Wellcome Trust Funding
  - North America Travel Fund
- Physical Science of Imaging in the Biomedical Sciences DTC
  - Dr. Iain Styles
  - EPSRC funding

Northwestern University, Chicago, Illinois

- Center for Advanced Microscopy and Nikon Imaging Center
  - Dr. Joshua Rappoport, Dr. Constadina Avantitis, Dr. Farida Korobova
  - Dr. Wilson Liu, Lennel Reynolds

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Thank you!

The research leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 691095.

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