Super resolution microscopy to unravel complex medicine-induced changes in the protein nano environment

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Background

Super resolution microscopy (Stochastic Optical Reconstruction Microscopy: STORM) enables a resolution of <30nm. This enhancement allows for visualisation and improved understanding of what is happening at the sub-micrometre level. Here, we are using this technique at the forefront of drug discovery to determine the mechanism of action of a novel complex medicine.

Apoferritin, the protein component of human ferritin, is crucial for iron storage but has significant potential as a targeted drug delivery carrier if the iron is removed leaving an 8 nm diameter hollow core.[1]

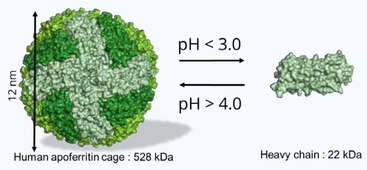


Figure 1: Human apoferritin cage is composed of 24 subunits, which reversibly assemble dependent on pH.[2]

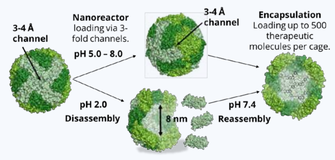
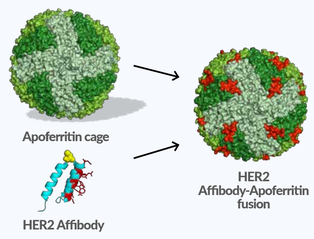


Figure 2: Apoferritin cages can encapsulate up to 500 therapeutic molecules through two methods; 'Nanoreactor' and 'Reassembly'.

HER2 is a receptor tyrosine kinase. With no known ligand, activation occurs through homodimerization or heterodimerization with EGFR, HER3 or HER4. Whilst internalisation inactivates HER2 receptor signalling.

Downstream signalling, through the Ras-Raf-MAPK and PI3-K/AKT pathways, increases cell proliferation and inhibits apoptosis.

Overexpression of HER2 occurs in ~25% of breast cancers and is associated with an aggressive phenotype. Current treatment, Trastuzumab (Herceptin®, Roche-Genetech), is associated with a low response rate and resistance can arise within 6 months of treatment.



Neil Thomas's lab have generated a HER2 Affibody-Apoferritin fusion

Each apoferritin subunit is fused to an affibody so the nanocage can potentially bind up to 24 HER2 receptors simultaneously (Trastuzumab can only bind ≤2 HER2 per antibody).

The HER2 affibody binds to an alternative site to Trastuzumab on the HER2 receptor.

Aims:

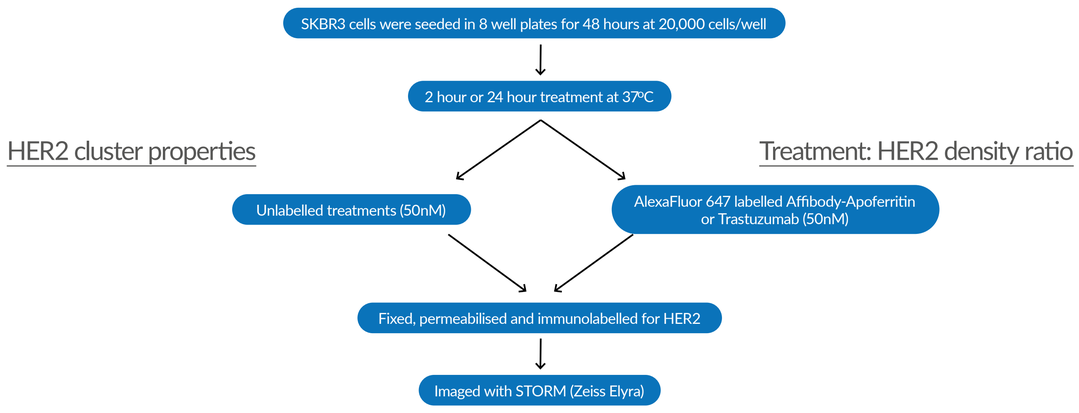
1. To determine the internalisation of Affibody Apoferritin conjugate in breast cancer cell lines
2. To compare HER2 cluster spatial properties following treatment
3. To examine treatment and receptor densities in different cellular regions

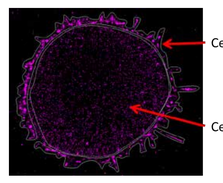
Methods

Affibody Apoferritin Internalisation in breast cancer cell lines

Breast cancer cells expressing different levels of HER2 were seeded in 96-well plate as 20,000 cells/well and incubated at 37°C incubator overnight. The next day, the cells were treated with Alexa647 labelled HER2 Affibody-Apoferritin (AA), Affibody or Trastuzumab. After 24h, the cells were washed with PBS and fixed with 4% PFA. Afterwards, the cells were imaged in Opera Phenix Plus HCS System (Perkin Elmer) using 63x water immersion objective. The acquired images were analysed using Harmony software to evaluate the number of Alexa647 spots inside the cells.

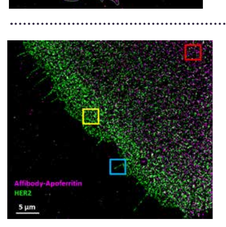
STORM imaging to determine effects of treatment





HER2 clusters were determined using a density-based clustering algorithm (DBSCAN)

Regions of interest were drawn around the membrane protrusions (cell edge) and non-protruding membrane on the coverslip (cell body)

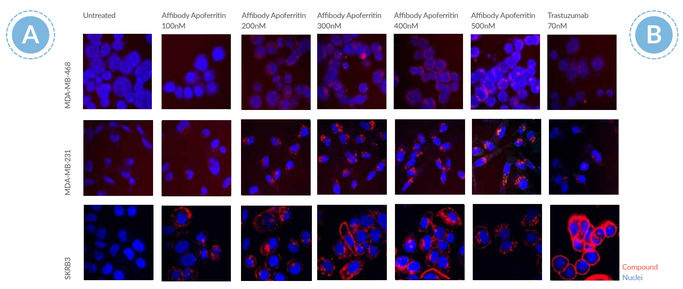


The density ratio of localisations of treatment to HER2 was calculated for different cellular regions.

Regions of interest:

Filopodia | Edge (not protruding) | Centre of cell body

Affibody Apoferritin Internalisation in breast cancer cell lines



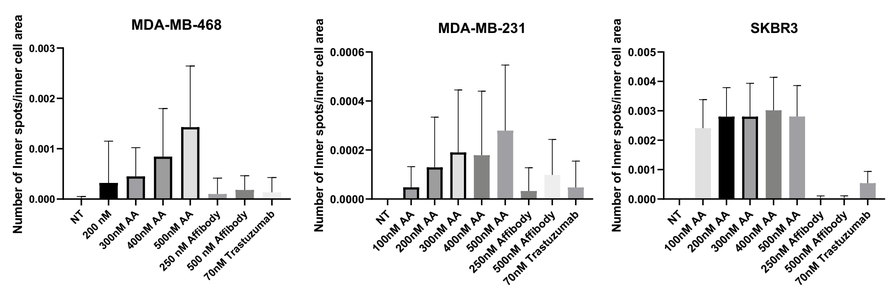
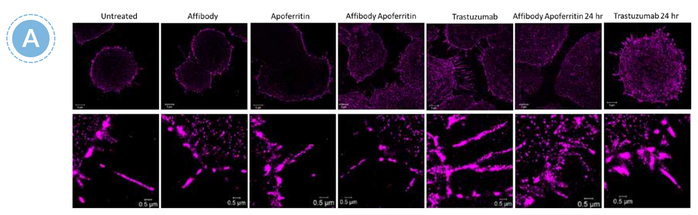
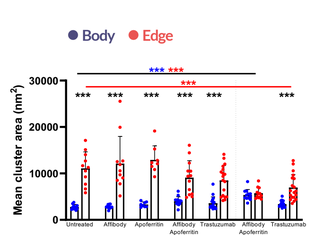


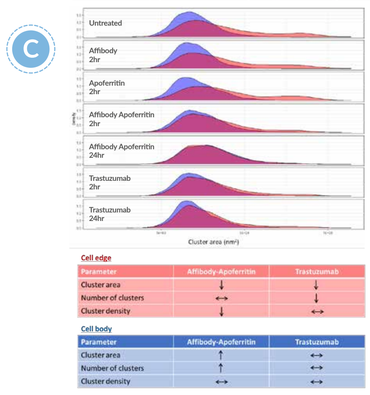
Figure 3: Breast cancer cell lines expressing different levels of HER2 were treated with Alexa647 labelled Affibody Apoferritin conjugate or Trastuzumab for 24 hours and the number of internalised particles were determined. MB-468 (HER2 ~zero receptors); MB-231 (HER2 normal ~20K); SKBR3 (HER2 + ~2-3 million receptors per cell).

HER2 Affibody-Apoferritin conjugate is internalised in breast cancer cell lines

HER2 cluster spatial properties







Cell edge

Parameter	Affibody-Apoferritin	Trastuzumab
Cluster area	↓	↓
Number of clusters	↔	↓
Cluster density	↓	↔

Cell body

Parameter	Affibody-Apoferritin	Trastuzumab
Cluster area	↑	↔
Number of clusters	↑	↔
Cluster density	↔	↔

Figure 4.

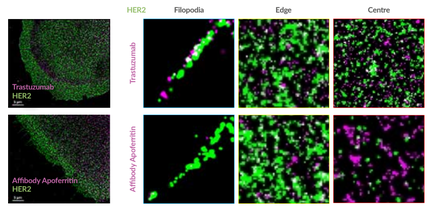
A: Super resolution images of HER2 in SKBR3 cells.

B: Mean HER2 cluster area, cluster density and number of clusters in each treatment group. N = 8-19 cells, 2 repeats.

C: Density distribution histogram of HER2 cluster area.

HER2 cluster spatial properties were altered following 24hr treatment

Treatment and HER2 in different cellular regions



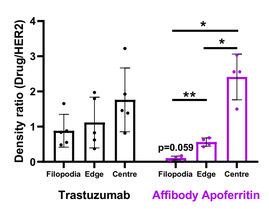


Figure 5: A: Dual-colour super resolution images of SKBR3 cells co-labelled for treatment and HER2. Different cellular regions were identified for analysis. B: Mean density ratio of treatment to HER2. N = 4-5 cells

Affibody Apoferritin density greatly reduced in 100-200nm filopodia compared to large-scale cellular regions

Summary

The results here show that the Affibody Apoferritin fusion has great potential as a therapeutic treatment of HER2 positive breast cancers.

Affibody Apoferritin:

1. Internalised in three breast cancer cell lines with variable HER2 expression
2. Reduced HER2 cluster size in the cell membrane protrusions after 24 hour treatment
3. Lower density ratio in the filopodia warrants further mechanistic investigation

Super resolution microscopy enabled an unprecedented ability to resolve the cellular actions of novel complex medicine together with the target receptor and identified potential mechanistic differences within the cellular nano environment.

Medicines Discovery Catapult (MDC) Funded by Innovate UK, MDC is national centre set up to help UK SMEs, biotechs, academics and innovators with access to cutting edge technology, expertise, data and networks they need to progress their drug discovery programs. Through collaborative programmes of R&D we are tackling the most challenging issues in drug discovery, addressing systemic problems and bottlenecks and using innovative technologies to enable "fast-to-patient" medicines discovery.

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References

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