Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information for Selective killing of cells triggered by their mRNA signature in the presence of smart nanoparticles

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S-I General

Ultrapure water (18 $M\Omega cm^{-1}$) was used throughout unless mentioned otherwise. All glassware was cleaned with aqua regia and rinsed with ultrapure water prior to any experiments. Commercially available reagents and solvents were used from the following suppliers throughout the experiments without further purification unless stated otherwise: Sodium tetrachloroaurate (III) dihydrate, trisodium citrate, sodium phosphate monobasic, sodium phosphate dibasic, Bis(p-sulfonatophenyl)phenyl phosphine dihydrate dipotassium salt (BSPP) and agarose were purchased from Sigma-Aldrich.

Primary antibodies (all from mouse host) for Vimentin (VIM 13.20) were purchased from Sigma Aldrich. Primary antibodies for Desmocollin 2+3 (7G6) and Keratin 8/18 (DC10) as well as secondary antibodies (goat, anti-mouse) labelled with Alexa Fluor 568 were purchased from ThermoFisher Scientific.

The differentiated SV-40 transformed bronchial epithelial cell line 16HBE, was a donation from Dr. D.C. Gruenert (University of California, San Francisco, CA).[1] Human foetal lung fibroblasts (MRC-5) were obtained from Sigma Aldrich. Cells were utilized up to passage 40.

UV – visible spectra were recorded on a Cary 100 UV- Vis spectrophotometer over a range of 400–800 nm. TEM images were obtained on a FEI Tecnai12 Transmission Electron Microscope operating at an accelerating voltage of 80 or 120 kV. Sample preparation involved deposition and evaporation of a specimen droplet onto a grid (carbon-copper coated 400 mesh). Solvent evaporation was carried on an Eppendorf Concentrator. Polyacrylamide gels were visualised under short-wave UV irradiation using a Syngene G:Box gel imager and GeneSnap software. All glassware and stirrer bars were rinsed with Aqua Regia followed by Milli-Q water.

S-II Cell culturing

16HBEs and MRC-5s were cultured in Corning cell culture flasks using Modified Eagle's Medium (MEM, Gibco) including 2mM L-glutamine (Gibco), supplemented with 100 U/ml penicillin/streptomycin (Gibco), 1 mL nystatin (Gibco) and 10% foetal bovine serum (FBS, Sigma Aldrich). Cells were kept at 37 °C, 5 % CO_2 atmosphere in a Sanyo CO_2 incubator (model MCO-17AI). Cells were grown to 90% confluency and then passaged once a week.

a. Immunofluorescent labelling

To highlight the presence of Vimentin, Desmocollin and Keratin 8 proteins in the model epithelial and mesenchymal cell lines, immunofluorescent labelling was performed. Cells were grown on square glass coverslips in 6-well plates. Once cells were ~90 % confluent, they were washed 3 × with PBS followed by fixation in ice cold methanol. Subsequently, cells were washed 3 × with phosphate buffer (0.1 M) followed by a 30 min blocking in phosphate buffer containing 1 % BSA. Afterwards cells were incubated with primary antibody (anti-Vimentin, anti-Desmocollin, anti-Keratin 8/18) for 45 min, followed by three washing steps with phosphate buffer containing 1 % BSA (5 min each). Cells were then incubated with secondary antibody (goat, anti-mouse, labelled with Alexa Fluor 568) for 45 min, followed by an additional three washing steps with phosphate buffer (0.1 M). Cells were then incubated with DAPI for 15 min followed by three washing steps with phosphate buffer (0.1 M). Coverslips were then mounted in Mowiol containing Citifluor on glass slides and stored at 4 °C. Imaging was carried out on a Leica SP8 confocal microscope using a × 63 oil immersion objective.

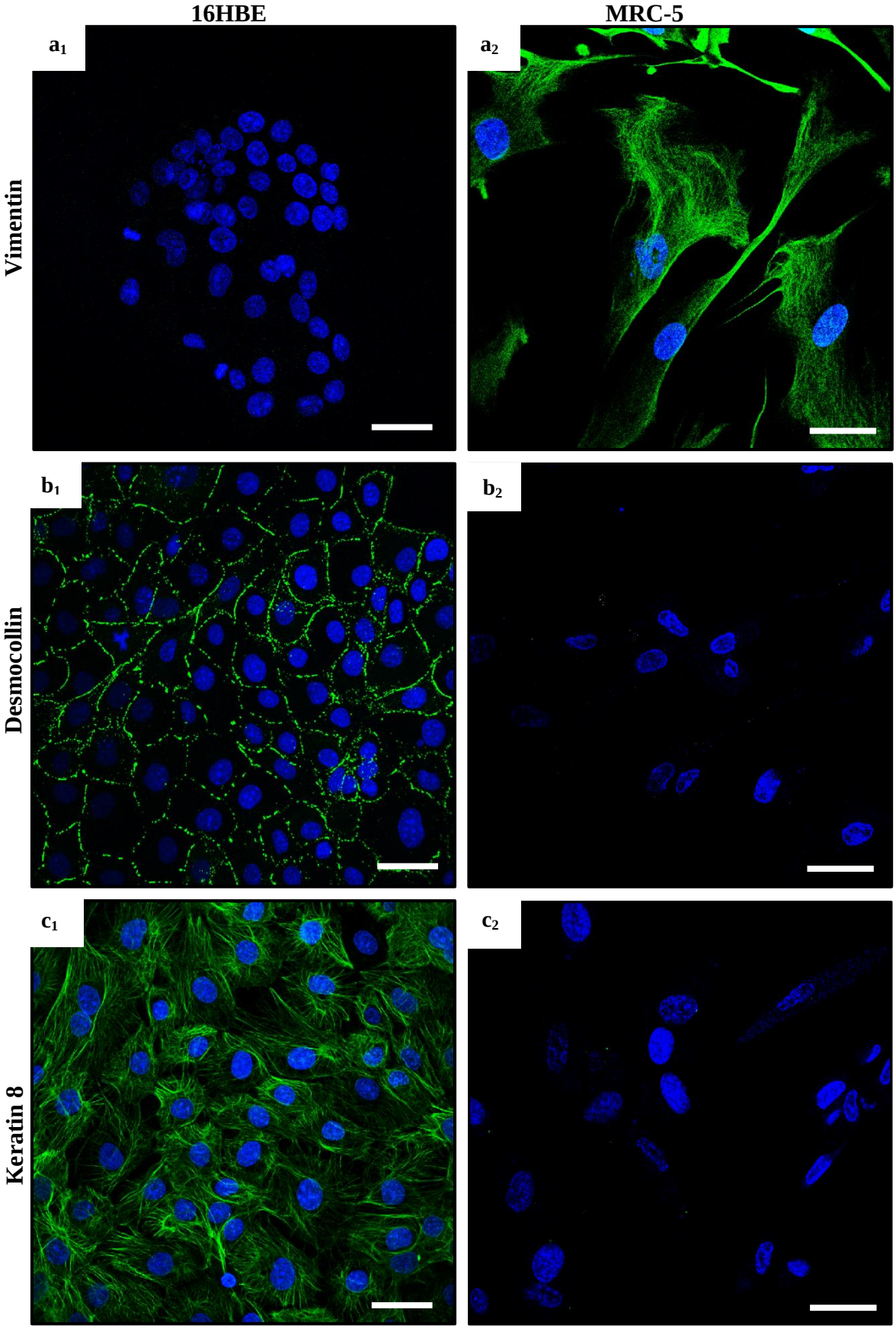


Figure S1. Immunofluorescent labelling of cells. 16HBE (1) and MRC-5 cells (2) were analysed for the expression of (a) Vimentin, (b) Desmocollin and (c) Keratin 8. Nuclei were stained with DAPI (blue). Secondary antibodies are labelled with Alexa 568 (green). Scale bars are 30µm.

Confocal images show that only the mesenchymal MRC-5 cells exhibit expression of the intermediate filament protein Vimentin expression (**Fig. S1a**). Contrastingly, Desmocollin and Keratin 8 expression was only observed in epithelial cells (**Fig. S1b** and **c**).

S-III Synthesis of 13 nm AuNPs

Gold nanospheres were synthesised according to the established literature protocol first reported by Turkevich[2]. In detail, an aqueous solution of sodium tetrachloroaurate (100 mL, 1 mM) was brought to the boil under stirring. Once boiling, a hot aqueous solution of trisodium citrate (5 mL, 2% wt/V) was added to the gold solution. The solution was then stirred under boiling for an additional 15min and subsequently allowed to cool to room temperature under stirring. To exchange the citrate ligand with bis(p-sulfonatophenyl)phenyl phosphine dihydrate dipotassium salt (BSPP) 20 mg of BSPP were added to the solution [3]. After 4 h of stirring, brine was used to induce particle aggregation *via* charge screening. Following two rounds of centrifugation (5000 rpm, 10 min), decantation/re-dispersion and sonication, particles were finally re-dispersed in Milli-Q water and purified by filtration (0.2 µm syringe filter, VWR). Particles were stored at 4 °C prior to further functionalization.

S-IV Design and synthesis of oligonucleotides

a. Design of sense and flare oligonucleotides

As a proof of concept, initially a nano-probe was designed capable of detecting all mature mRNAs. By displaying a sense sequence consisting of polyT and a short polyA flare strand, these probes were designed to detect all mature mRNAs *via* their characteristic polyA tail[4].

mRNA sequences mRNA for Vimentin, Desmocollin (pan) and Keratin 8 were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/nucleotide/). Sequences were then analysed

for alignments with other sequences (i.e. similarities) using the NCBI's **B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST) (www.ncbi.nlm.nih.gov/BLAST.cgi). This tool is an algorithm constructed to compare sequence information such as amino acids, proteins or nucleotides using published databases[5]. Here the nucleotide-specific blastn was utilized. The following settings were applied: Database: RefSeq RNA; Entrez Query: all[filter] NOT predicted[title]; Species: Homo sapiens; Expect threshold: 10; Match/Mismatch scores: 2/-3. Appropriate sequence targets were then chosen with the following criteria: Length of sense strand: 20-23 bases, melting temperature ~60 °C, GC content < 50 %, E value < 0.05, E value of nearest match > 1. Accordingly the flare strand was designed complementary to the sense strand with the following criteria: Length of flare strand: 10-12 bases, melting temperature ~40 °C.

Table S1. Oligonucleotide sequences. S1: 3´-Thiol modifier 6 S-S (CPG resin from Glen Research) ($C_2H_4OC_3H_6S$-$SC_3H_6OC_2H_4$), S2: 5´ -Thiol modifier C6 S-S (Phosphoramidite from link technologies) (C_6H_{12} S-S C_6H_{12}).

Name	DNA sequences (5´-3´) and modifications
Vimentin Sense	**Cy3**-CTT TGC TCG AAT GTG CGG ACT TAA AAA AAA-**S1**
Vimentin Flare	**Cy5**-AAG TCC GCA CA
Vimentin perfect target	AAG TCC GCA CAT TCG AGC AAA G
Vimentin 1 mismatch target	AAG T**T**C GCA CAT TCG AGC AAA G
Vimentin 2 mismatch target	AAG T**T**C GA**C** CAT TCG AGC AAA G
Desmocollin Sense	**FAM**-TGA GTA AAA CTG TGC CAC TCC GAA AAA AAA-**S1**
Desmocollin Flare	**Cy3**- C GGA GTG GCA CA
Desmocollin perfect target	CGG AGT GGC ACA GTT TTA CTC A
Desmocollin 1 mismatch target	CGG AGT G**T**C ACA GTT TTA CTC A
Desmocollin 2 mismatch target	CG**A** AGT G**T**C ACA GTT TTA CTC A
Keratin 8 Sense	**FAM**-GGT GGT CTT CGT ATG AAT ACA AAA AAA A-**S1**
Keratin 8 Flare	**Cy3**-GTA TTC ATA CGA AG
Keratin 8 perfect target	GTA TTC ATA CGA AGA CCA CC
Keratin 8 1 mismatch target	GTA TTC ATA **T**GA AGA CCA CC
Keratin 8 2 mismatch target	GTA **C**TC ATA **T**GA AGA CCA CC
General mRNA Sense	**S2**-AAA CGG GCT TTT TTT TTT TTT TTT TTT TTT TTT T-**Cy3**
General mRNA Flare	AAA AAA AAA AAA GCC C-**Cy5**
Nonsense mRNA Sense	**S2**-AAA ATT GTC AGA AAG CCA TAT GG TA-**Cy3**
Nonsense mRNA Flare	GCT TTC TGA CAA-**FAM**

b. Oligonucleotide synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/ RNA synthesizer using a standard 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the

automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 40 s, and the coupling time for the Cy3, Cy5 and 5'-thiol Modifier C6 S-S phosphoramidite monomers was extended to 600 s. 3′-Thiol modifier 6 S-S CPG synthesis columns (Glen Research) were used for the introduction of the 3′-disulphide moiety into oligonucleotides. Cleavage of the oligonucleotides from the solid support and de-protection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. Cy5-labelled oligonucleotides were made with acetyl dC and dmf-dG monomers so that de-protection time could be reduced to 1 hr to prevent degradation of Cy5. The oligonucleotides were purified by reversed-phase HPLC on a Gilson system using an XBridgeTM BEH300 Prep C18 10 µM 10×250 mm column (Waters) with a gradient of acetonitrile in ammonium acetate (0% to 50% buffer B over 30 min, flow rate 4 mL/min), buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate, pH 7.0, with 50% acetonitrile. Elution was monitored by UV absorption at 300 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare). Mass spectra of oligonucleotides were recorded in ES- mode in water on a Bruker microTOFTM II focus ESI-TOF MS instrument, and in all cases confirmed the integrity of the sequences.

S-V Synthesis and characterization of nano-probes

a. Functionalization of AuNPs with oligonucleotides

13 ± 1 nm AuNPs were modified with a shell of oligonucleotides according to literature procedures. The procedure involved a slow 'salt ageing' process[6], required to screen repulsive negative charges between DNA phosphate backbones and the particle surface, thus

ensuring a high oligonucleotide surface loading. In brief, BSPP-coated AuNPs (1 mL, 10 nM) were incubated with disulfide modified DNA (3 nmol) overnight to allow for DNA adsorption onto the nanoparticle surface, forming a low density monolayer.[7] BSPP (1 mg/20 μL, 10 μL), phosphate buffer (0.1 M, pH 7.4) and sodium dodecyl sulfate (SDS) (10 %) were then added to achieve final concentrations of 0.01 M phosphate and 1 % SDS respectively. The addition of BSPP resulted in the conversion of the disulfide terminus on the oligonucleotide strands into thiols. Six additions of a NaCl solution (2 M) over a period of 8 h were required to achieve a final salt concentration of 0.15 – 0.2 M. The resulting DNA-coated AuNPs were then purified by three subsequent centrifugation (16400 rpm, 10 min) and decantation/re-dispersion steps. Finally, full characterized conjugates were stored in sterile PBS buffer at 4 °C. The number of oligonucleotides per AuNP were determined as follows: to a solution of DNA-coated AuNPs of a known concentration (200 μL) was added a solution of KI/I_2 (ratio of I_2 to KI = 1:6, 300 μL, 34 mM).[8, 9] A colour change from red to yellow indicated the dissolution of colloidal gold. The solution was made up to 1 mL with water and subsequently loaded onto a NAP10 desalting column. After the solution had entered the column, it was eluted with water (1.5 mL) and the product was collected. The oligonucleotide concentration was determined by UV-vis spectroscopy. The degree of oligonucleotide-loading was hence found to be ~ 120 ± 2 DNA strands per AuNP.

b. Nano-probe Synthesis

DNA-AuNP conjugates (in PBS) prepared by the salt ageing method were mixed with the flare strand and heated to 70 °C for 5 min, followed by slow, gradual cooling to room temperature overnight to allow for hybridisation to occur. Fully assembled nano-probes were then purified by triple centrifugation (16400 rpm, 15 min), decantation/re-dispersion and

sonication. Nano-probes were finally re-suspended in sterile PBS buffer (100 μL) and immediately used for cell culture experiments.

For implementation of Doxorubicin, nano-probes (in PBS) were incubated with an excess of drug (1 mg/4 mL, Sigma Aldrich) and varying amounts of flare strands. The mixture was then heated briefly to 70 ºC and subsequently left to slowly and gradually cool to room temperature. Doxorubicin-loaded nano-probes were then purified at least three times (or until no Doxorubicin fluorescence (excitation at: 480 nm, emission scan: 500-800 nm) could be observed in the supernatant) by centrifugation (16400 rpm, 20 min) and re-dispersion in sterile PBS. Assembled probes were then immediately used in further cell culture experiments.

c. Oligonucleotide fluorescence melting analysis

Fluorescence melting analysis allows for monitoring of flare strand (de-)hybridisation as well as for determination of the melting temperature of nano-probes. Hence, assembled nano-probes in PBS (150 μL, 2.5 nM) were slowly heated in a low volume Quartz cuvette at a rate of 0.1 °C/min and a temperature range from 25 °C – 70 °C in a Cary Eclipse Fluorescence Spectrophotometer. Readings were taken at a rate of 0.1 °C/min (excitation wavelengths: 543 nm (Cy3) or 635 nm (Cy5) and emissions wavelengths: 563 nm (Cy3) or 662 nm (Cy5)).

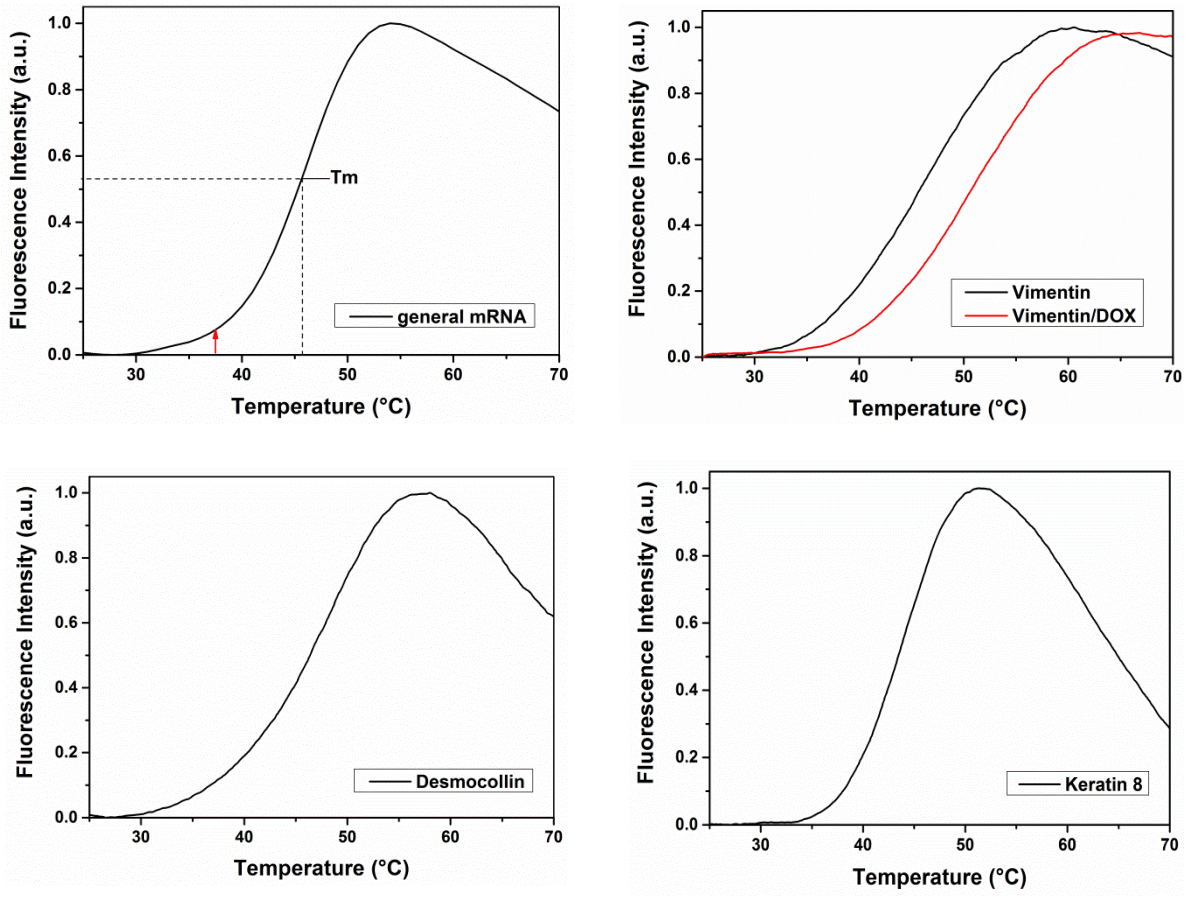


Figure S2. Fluorescence melting curves of general mRNA, Vimentin (or Vimentin-DOX), Desmocollin and Keratin 8 nano-probes. To determine the nano-probes' melting temperatures and analyse sense-flare hybridisation, all fully assembled nano-probes were heated to 70 °C and the flare strand fluorescence was monitored.

d. Determination of target specificity

Being designed for specific intracellular mRNA detection, it is vital for the nano-probes to display excellent target specificity. Thus their response to perfect and mismatched targets was assessed. In brief, assembled nano-probes in PBS (150 μL, 2.5 nM) were incubated at 37 °C. Target sequences (prefect target, 1 mismatch target or 2 mismatch target) were then added and fluorescence readings were taken immediately until a plateau was reached. Fluorescence

spectra were recorded, exciting at 543 nm (Cy3) or 635 nm (Cy5) and scanning the emission over a range of 550-800 nm (Cy3) or 650-800 nm (Cy5).

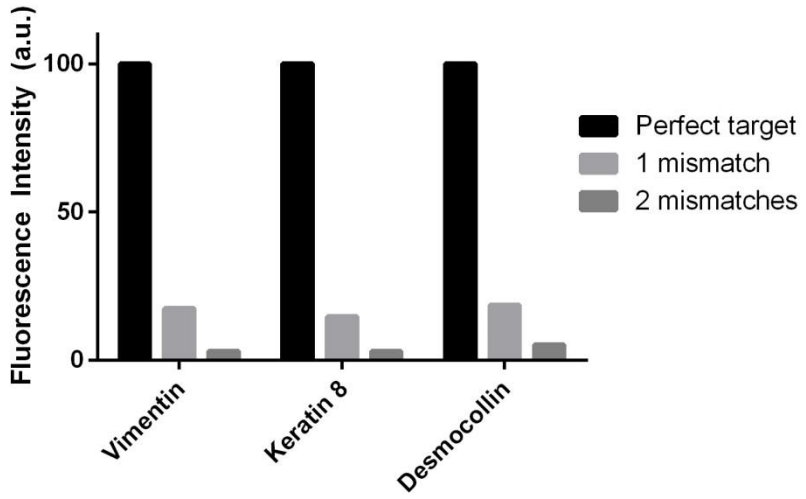


Figure S3. Relative fluorescence signal of nano-probes in response to perfect and mismatched targets. Nano-probes were exposed to different targets containing one, two or no mismatches. Their response in the form of flare strand fluorescence was analysed and results were plotted, normalised to the maximum amount released.

As illustrated in Figure S3, all nano-probes exhibited excellent target specificity with a relative fluorescence signal of only 20 % upon incubation with a one-mismatch target, compared to the perfect target.

e. Stability assays

Prior to live cell applications, it was imperative to ensure that our probes exhibit excellent bio-stability. The two main factors affecting intracellular stability are degradation by nucleases and the displacement of oligonucleotides from the AuNP surface by competitive conjugation of glutathione through its cysteine side chain.

e.i Glutathione assay

Assembled general mRNA nano-probes in cell culture medium MEM (150 μL, 1 nM) were incubated at 37 °C with glutathione (5 mM final concentration). The fluorescence of the sense strand (Cy3) was monitored immediately for up to 48 h.

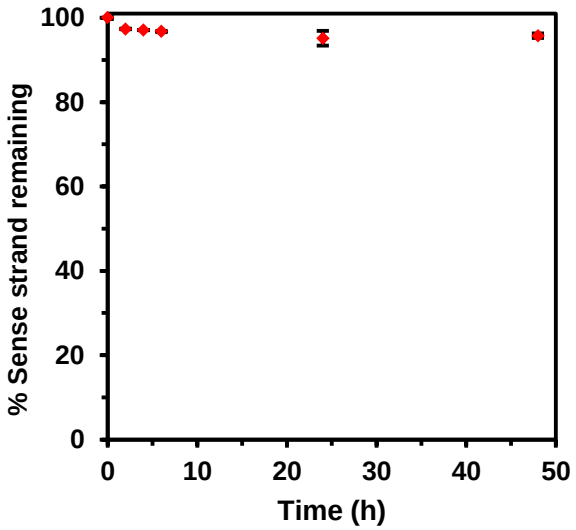


Figure S4. Determination of the amount of sense strand remaining on the gold nanoparticle core after incubation with Glutathione. The amount of functional sense strand remaining on the gold nanoparticle core after incubation with Glutathione for up to 24 h was determined by normalisation against 100 % release signal. The data shows that more than 90 % of sense strands remain conjugated after 18 h. Data are shown as mean ± SEM (n = 3).

e.ii Nuclease assays

For DNase I nuclease assays, assembled nano-probes (150 μL, 1 nM) in a buffer containing Tris-HCl (10 mM), $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.5 mM), pH 7.4 were incubated at 37 °C with DNase I (from bovine pancreas, Sigma Aldrich, 2U/L) and fluorescence of the sense strand (Cy3 or FAM respectively) was monitored immediately for up to 48 h. For DNase II nuclease assays, assembled nano-probes (150 μL, 1 nM) in artificial lysosomal fluid (ALF)[10] were incubated at 37 °C. DNase II (from porcine spleen, Sigma Aldrich) was then added (5

U/rxn) and fluorescence of the sense strand (Cy3 or FAM respectively) was monitored immediately for up to 48h.

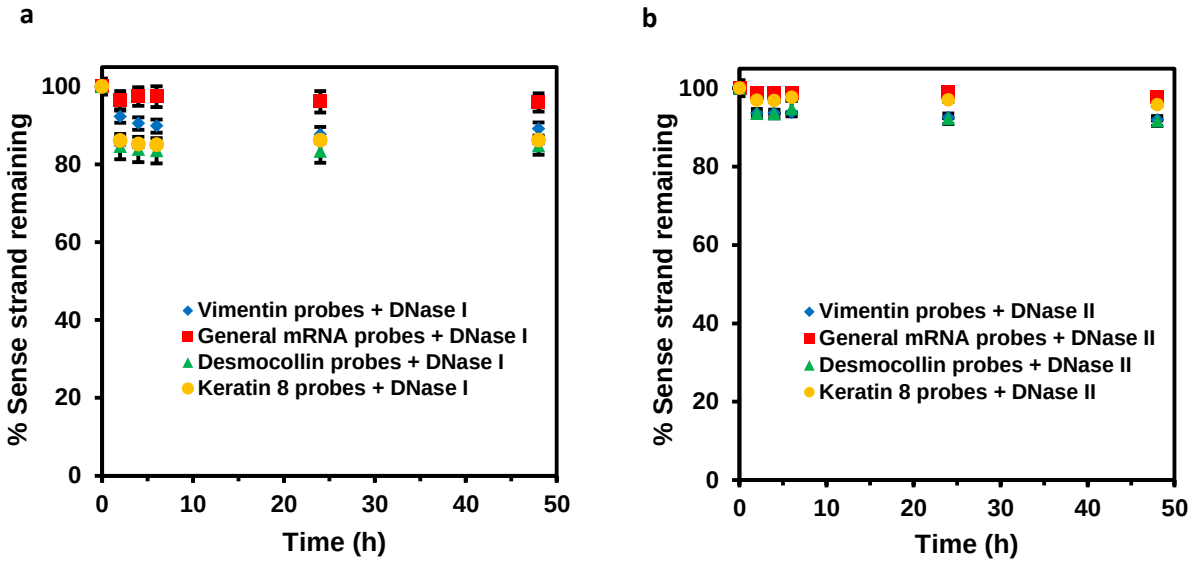


Figure S5. Determination of the susceptibility of nano-probes towards degradation by nuclease enzymes**.** The amount of functional sense strand remaining on the gold nanoparticle core after incubation with DNase I (a) or DNase II (b) was determined via fluorescence spectroscopy by normalisation against the 100 % release fluorescence signal. The data shows that none of the nano-probes were significantly affected within the time-frame and reaction conditions tested. Data are shown as mean ± SEM (n = 3).

e.iii Determination of Doxorubicin leaking from nano-probes

In order to an analyse the behaviour of Doxorubicin once loaded into the nano-probes, Vimentin probes (150 µL, 1 nM in PBS) were loaded with Doxorubicin (350 pmol). After 2 h, probes were purified 3× by centrifugation (16400 rpm, 10 min) and re-dispersion in PBS (150 µL). Subsequently the fluorescence of DOX in the sample was measured for up to 24 h.

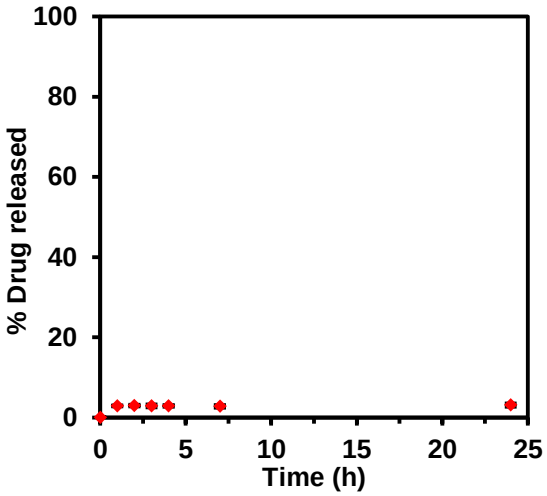


Figure S6. Determination of Doxorubicin leaking from Vimentin nano-probes. The amount of Doxorubicin released non-specifically from Vimentin nano-probes was determined via fluorescence spectroscopy (excitation: 480 nm, emission scan: 500-800 nm) and normalisation to the signal of fully released Doxorubicin (100 % release through DNA melting). Data are shown as mean ± SEM (n = 3).

e.iv Determination of Doxorubicin loading

Vimentin nano-probes (150 µL, 2.5 nM) in PBS, loaded with varying amounts of Doxorubicin were heated to 75 °C for 5 min. Probes were subsequently centrifuged at 16400 rpm for 10 min. Doxorubicin fluorescence in the supernatant was then recorded (excitation: 480 nm, emission scan: 500-800 nm). The drug-loading of each probe was determined from an appropriate calibration curve of doxorubicin fluorescence at 590 nm. From this it was determined that the average DOX loading was: 57 ± 4 for "60×" duplex, 73 ± 2 for "80×" duplex, 95 ± 5 for "100×" duplex and 112 ± 5 for "120×" duplex.

Table S2. Determination of Doxorubicin-loading per Vimentin nano-probe.

	"60x"				"80x"				"100x"				"120x"			
Measurement	Abs. (a.u.)	Conc. (nM)	No. of moles (pmol)	Equiv.	Abs. (a.u.)	Conc. (nM)	No. of moles (pmol)	Equiv.	Abs. (a.u.)	Conc. (nM)	No. of moles (pmol)	Equiv.	Abs. (a.u.)	Conc. (nM)	No. of moles (pmol)	Equiv.
1	5.89	129.74	19.46	51.89	8.15	179.52	26.93	71.81	10.96	241.41	36.21	96.56	13.36	294.27	44.14	117.71
2	7.48	164.76	24.71	65.90	8.83	194.49	29.17	77.80	11.67	257.05	38.56	102.82	13.01	286.56	42.98	114.63
3	6.22	137.00	20.55	54.80	7.92	174.45	26.17	69.78	9.88	217.62	32.64	87.05	11.67	257.05	38.56	102.82
SEM	0.48	10.67	1.60	4.27	0.27	6.02	0.90	2.41	0.52	11.46	1.72	4.58	0.52	11.34	1.70	4.54
Average	6.53	143.83	21.57	57.53	8.30	182.82	27.42	73.13	10.84	238.69	35.80	95.48	12.68	279.30	41.89	111.72

S-VI Interactions of nano-probes with cells

a. Incubation of cells with nano-probes for confocal imaging

Cells were grown on square glass coverslips in 6-well plates. Once cells were ~90 % confluent, media was exchanged with fresh media containing different types of nano-probes (2 mL, 1 nM) for 18 h. Afterwards cells were incubated with Hoechst 33342 (5 μL) for 1 h to stain DNA and nuclei. For imaging, the glass coverslip was removed and washed with PBS. It was then mounted on a glass slide with a drop of PBS in order to keep cells alive. The edges around the coverslip were sealed using double-sided tape thus creating a small 'incubation chamber'. All imaging was carried out on a Leica SP5 confocal microscope at 37 °C. Initial studies were focussed on the detection of all mature mRNAs using the general mRNA nano-probe. As discussed previously, these probes were engineered specifically to allow for the detection of all mature cellular mRNA via its characteristic polyA tail. Live cell confocal microscopy revealed bright intracellular fluorescence of the released flare strand in both the epithelial (16HBE, Fig S7a and S7c) and mesenchymal (MRC-5, Fig S7b) model cell lines tested. However, no significant fluorescence signal from the sense strand could be detected, suggesting that the sense strands anchored to the gold nanoparticle were not subject to intracellular degradation and the observed signal was indeed due to intracellular detection of mRNA. It is noteworthy that the localisation of the fluorescent signal is highly specific and appears to correspond to mitochondrial co-localisation (Fig. S7c). This finding was in agreement with a study conducted by Bao and co-workers, showing that 2'-deoxy and 2'-O-

methyl oligonucleotides labelled with Cyanine dyes co-localised with mitochondria, due to the mitochondrial membrane potential[11] as well as findings by Mirkin and co-workers[12]. We can therefore assume that the Cyanine-dye-tagged flare strand diffused away from the probe, towards the mitochondria, where it got trapped within the inner mitochondrial membrane, resulting in the observed fluorescence signal localisation.

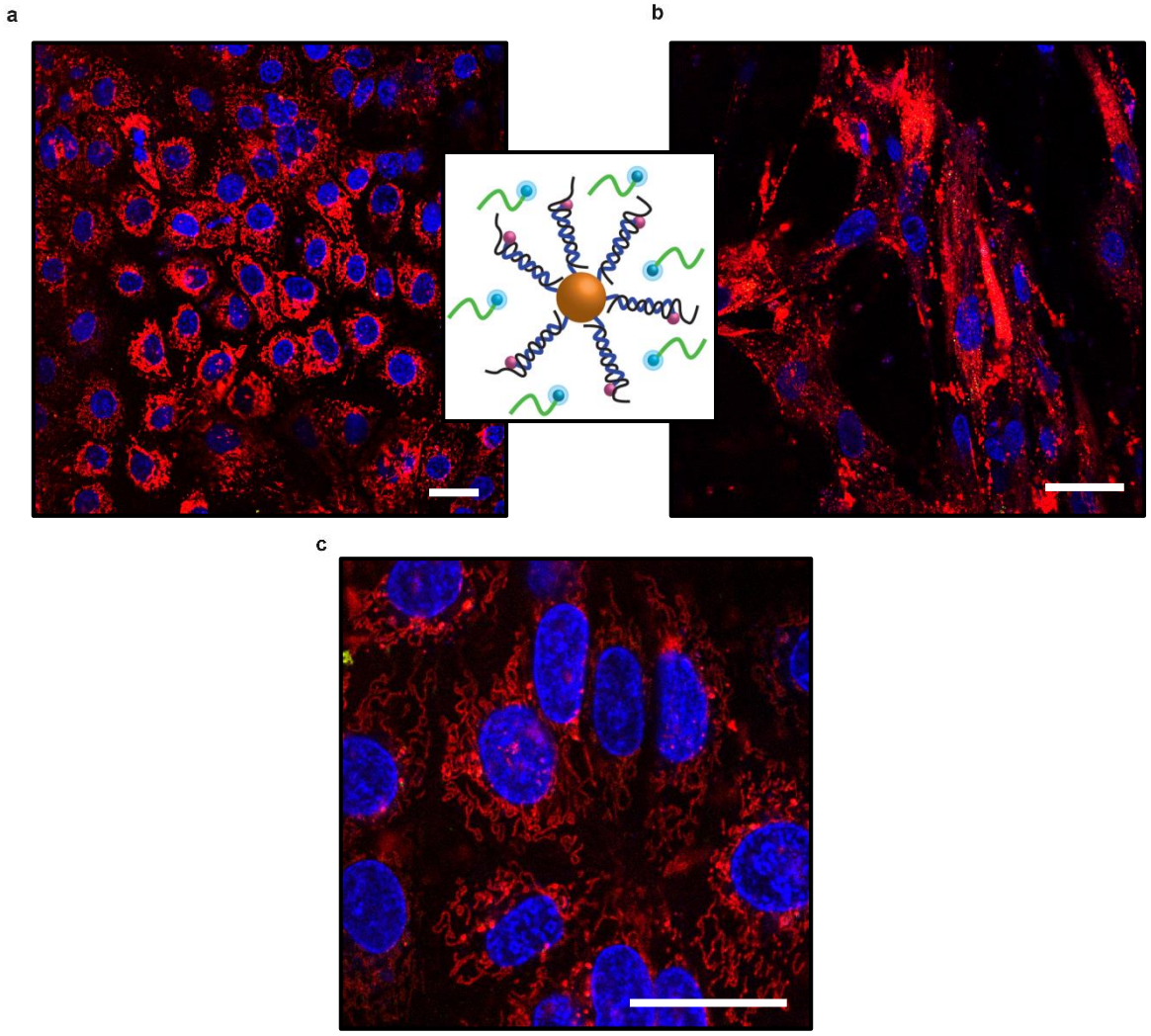


Figure S7. Live cell confocal microscopy images of cells incubated with the general mRNA nano-probe. (a) Model epithelial (16 HBE) and (b) mesenchymal (MRC-5) cell lines were incubated for a period of 18 h with our general mRNA nano-probes prior to visualisation of fluorescence by live cell confocal microscopy. (c) High magnification image showing mitochondrial association of the flare strand. Colour guide: Flare strand – red, sense strand – green, nuclear counter stain – blue. Scale bars are 30µm.

Secondly, as a negative control, we designed a 'nonsense' nano-probe, for which no mRNA target existed in the cell lines chosen. Live cell confocal microscopy revealed that no significant release of flare strand occurred in either cell line could be observed (Fig. S8). Furthermore no fluorescence signal of the anchored sense strand was visible, demonstrating the intracellular stability of the nano-probes.

b. Viability assays

Cells were seeded in 24-well plates to ~90 % confluency. Cells were then incubated with respective nano-probes (0.5 mL, 1 nM) for 18 h. The medium was subsequently removed and collected. Cells were washed with HBSS buffer followed by incubation with trypsin (350 μL, 0.25 % trypsin 0.01 % EDTA solution) to detach cells (5 min, 37 °C). Trypsin was then inhibited with cell culture media containing 10 % fetal bovine serum. Detached cells were then combined with the previously removed medium and subsequently pelleted by centrifugation at 1000 rpm for 5 min. The supernatant was removed and cells were re-suspended in MEM medium (10 μL). The cell viability was then assessed using the trypan blue dye exclusion assay. Staining in a 1:1 ratio with trypan blue resulted in non-viable cells displaying a blue coloured cytoplasm, whilst healthy cells retained a clear cytoplasm. Cells were then counted using a Neubauer haemocytometer and cell viability was evaluated. All viability assays were obtained from triplicates.

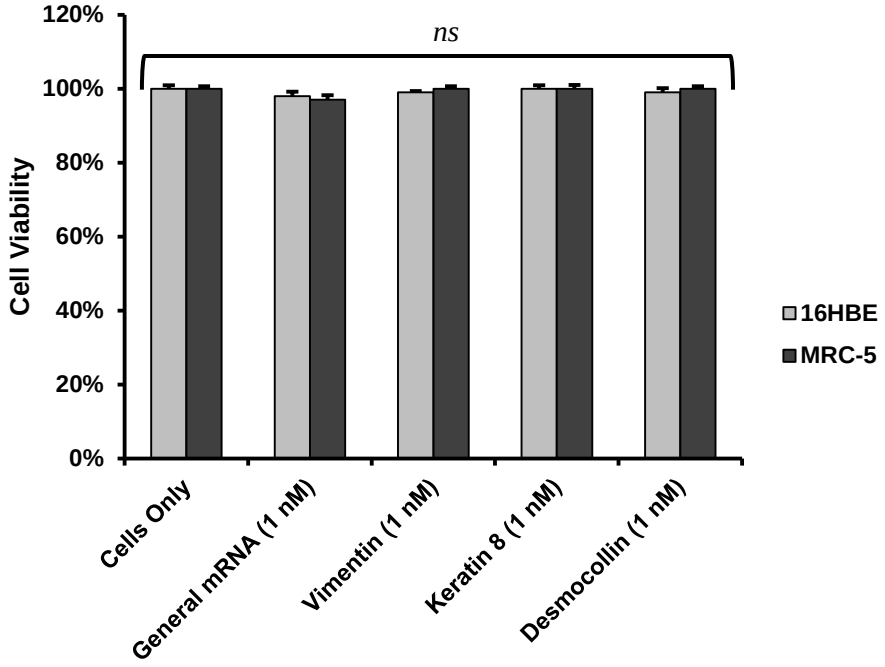


Figure S8. Viability assays of 16HBE and MRC-5 cells. Both model epithelial and mesenchymal cells lines were incubated with respective nano-probes (1 nM) for 18 h. Cell viability was determined using the trypan blue dye exclusion assay. Data are shown as mean ± SEM (n = 3). Data were analysed by a one-way ANOVA. *ns:* not significant.

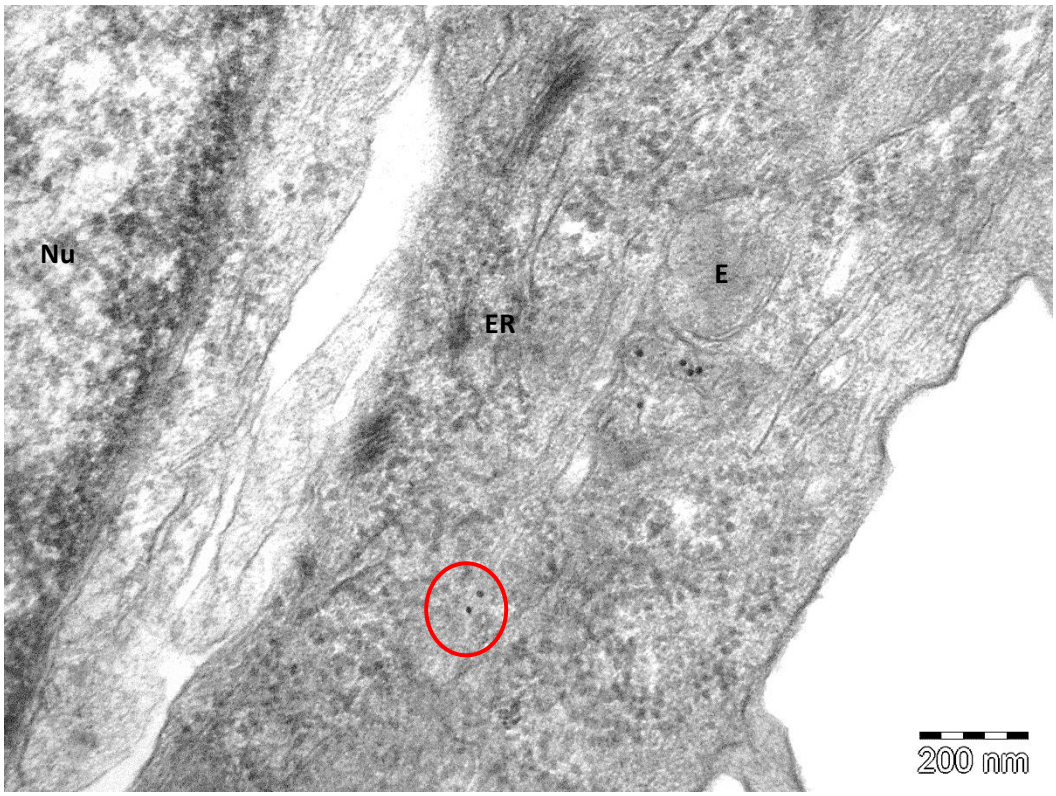
c. Ultra-thin sectioning of samples for transmission electron microscopy

To investigate the intracellular fate of nanoparticles, cells were embedded in resin, cut into ultrathin sections and visualised by transmission electron microscopy. Resin embedding ensures the preservation of cell structure and intracellular compartments and allows specimens to be viewed in small sections with highly detailed resolution. For this purpose, cells were seeded on cellulose transwell inserts in 12-well plates at cell concentrations of 1×10^5 cells/mL for 16HBE or 0.5×10^5 cells/mL for MRC-5 cells. Once cells had grown to ~90 % confluency they were incubated with general mRNA nano-probes for set amounts of time (1, 2, 4, 6 and 18 h) after which they were fixed for 1 h in 3% glutaraldehyde/4 % formaldehyde in 0.1 M piperazine-1,4-bis(2-ethanesulfonic acid (PIPES buffer 0.1 M, pH

7.2). Then three washing steps (10 min each) with PIPES buffer followed. Cells were subsequently treated with osmium tetroxide (1 % in PIPES buffer 0.1 M, pH 7.2) for 1 h. After a further three washing steps with PIPES buffer and one brief 30 s wash with deionised water, cells were stained with a 2 % aqueous solution of uranyl acetate for 20 min. The sample was then dehydrated by treatment with increasing % ages of ethanol solutions (30, 50, 70, 90 %) for 10 min each. N.B.: After treatment with 50 % ethanol, transwell inserts were cut from the plastic in order to avoid plastic dissolution in high % ethanol. From this point onwards transwell membranes were placed in a small glass vial. After treatment with 90 % ethanol, the sample was finally fully dehydrated by two consecutive washes in absolute ethanol (20 min each). In order to prepare for resin embedding, samples were then treated with acetonitrile for 10 min followed by 1:1 acetonitrile:SPURR resin overnight. The sample was subsequently fully emerged in pure SPURR resin for 6 h for complete infiltration in order to ensure the removal of acetonitrile. After 6 h the transwells were cut in half and each embedded in fresh SPURR resin in small embedding capsules and polymerised at 60 °C for 24 h. Resin blocks were then cut using a Leica RM 2255 ultramicrotome. Ultrathin (~100 nm) sections were collected on TEM grids (200 mesh) and visualised on a Hitachi H7000 transmission electron microscope (operating bias voltage of 75 kV).

For the investigation of intracellular particle location, five grids from three separate experiments per cell type were analysed (< 1700 particles per cell line). Results showed that whilst the majority of particles were located in vesicles (early and late endosomes) a small percentage of particles appeared to be located in the cytosol (16HBE total no. of particles counted: 1779; 3.60 % ± 0.73 % of particles were found within the cytosol. MRC-5 total no. of particles counted: 1834; 3.65 % ± 0.95 % of particles were found within the cytosol). Interestingly the percentage of particles inside the cytosol was found to be similar for both cell types, potentially suggesting a common mechanism.

a



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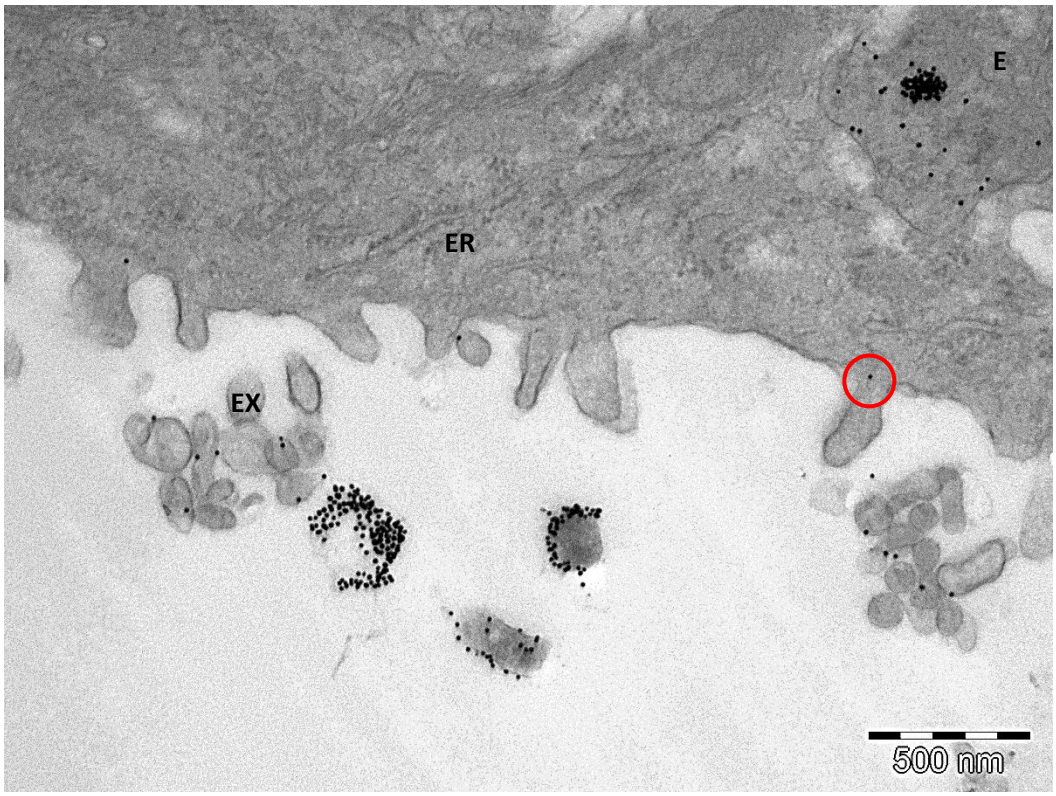

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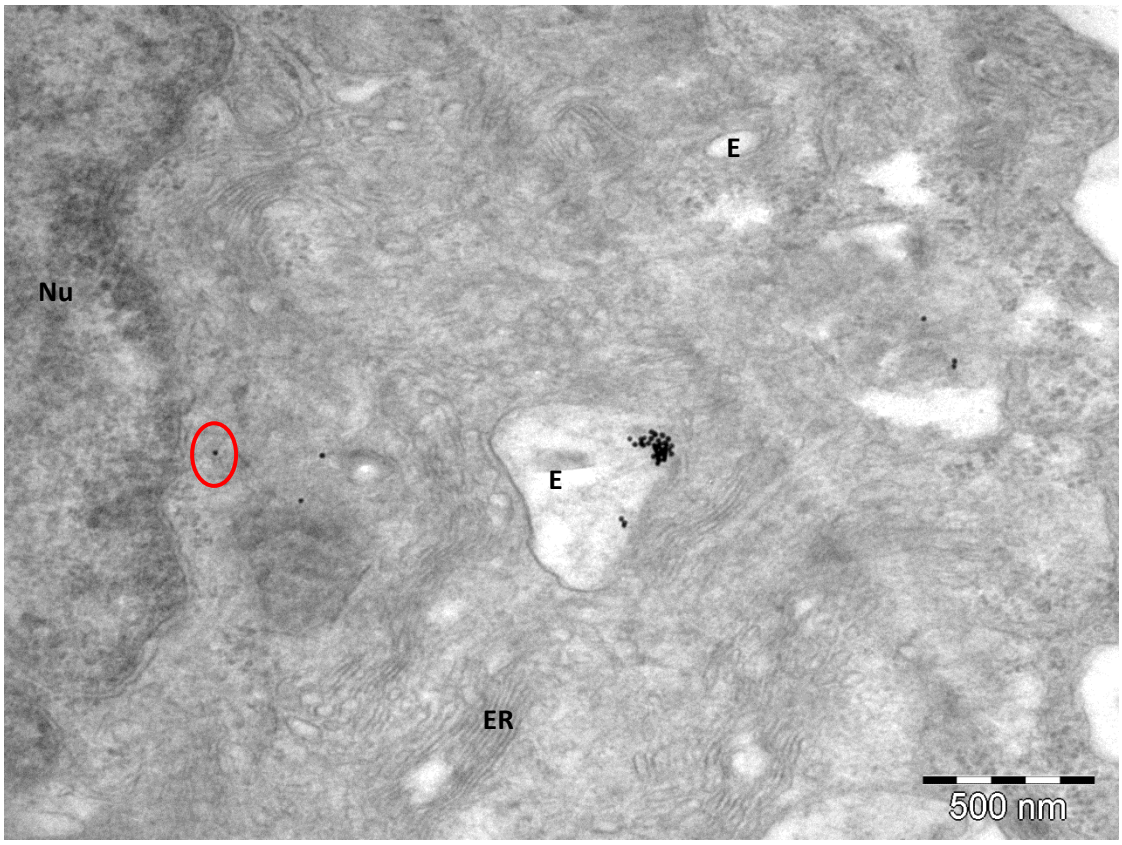

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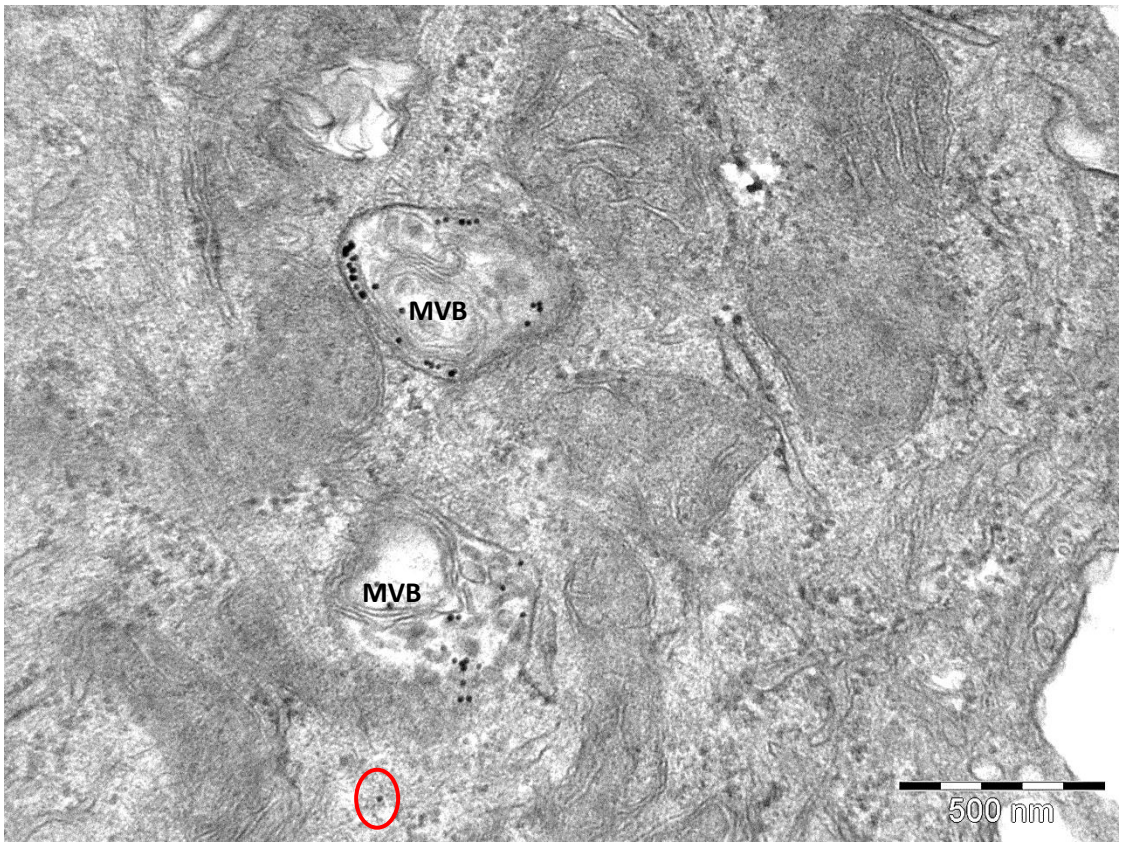

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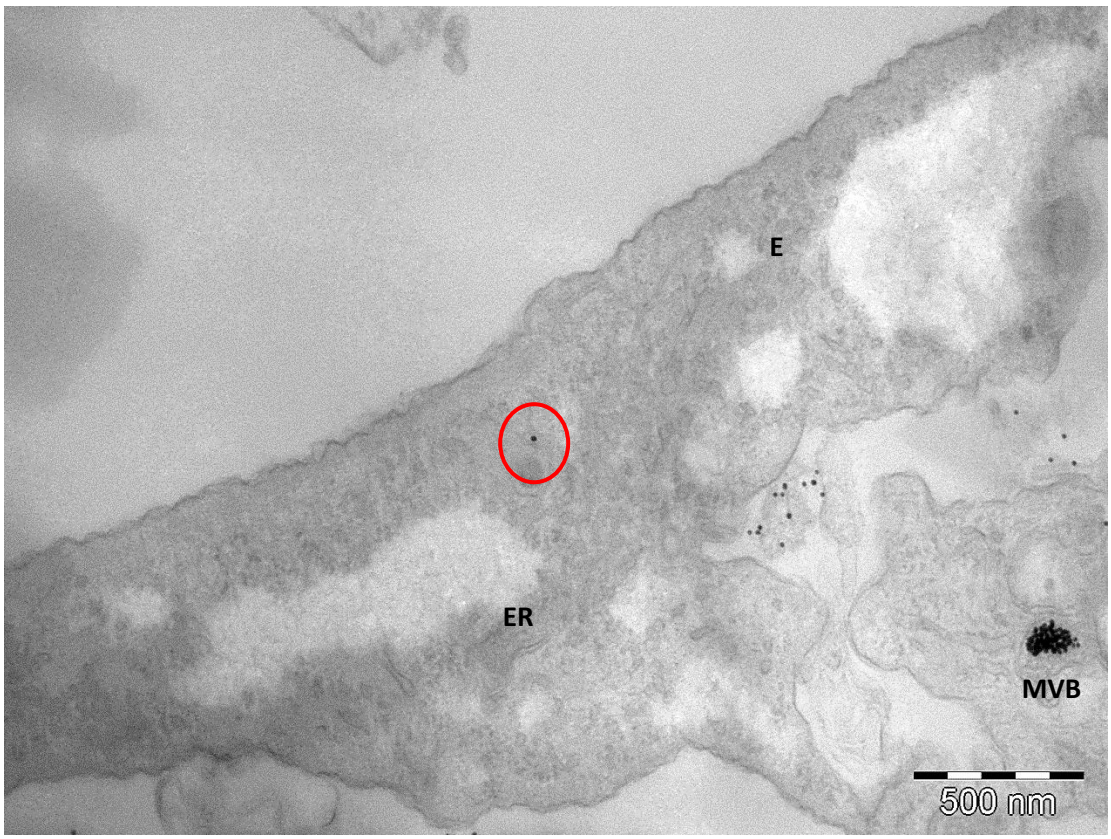

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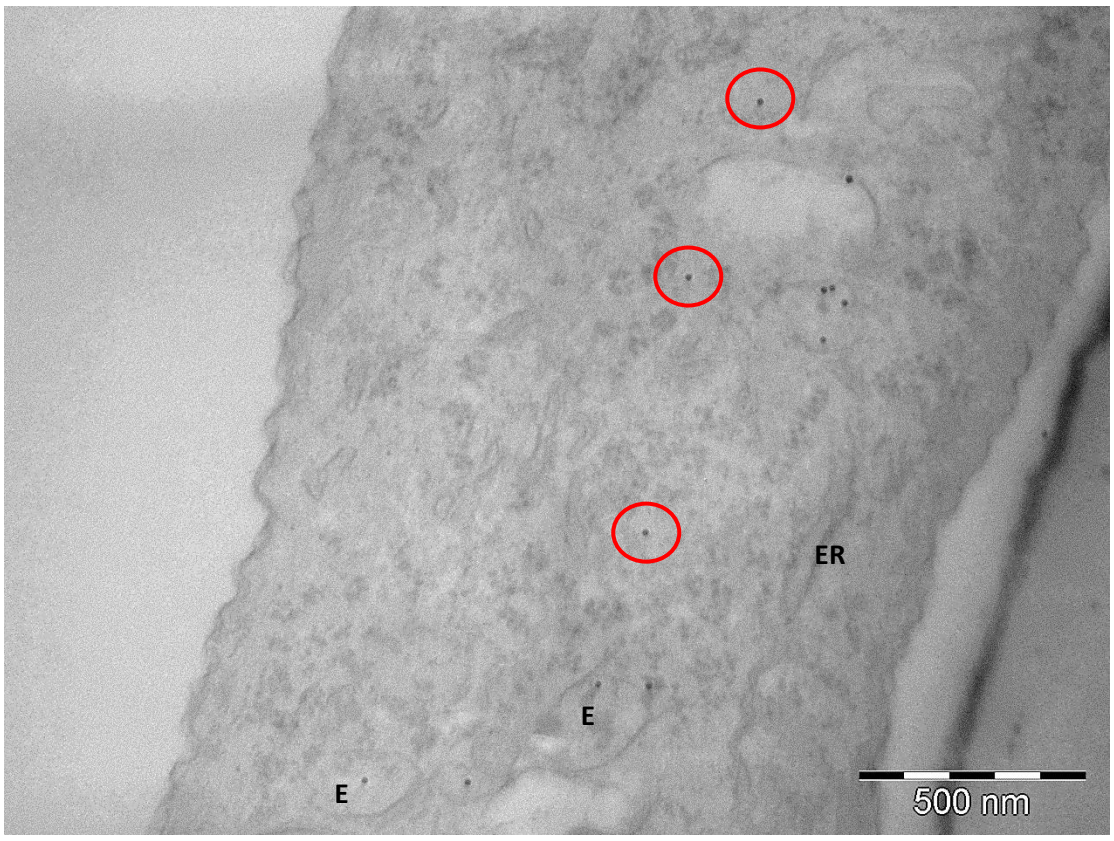

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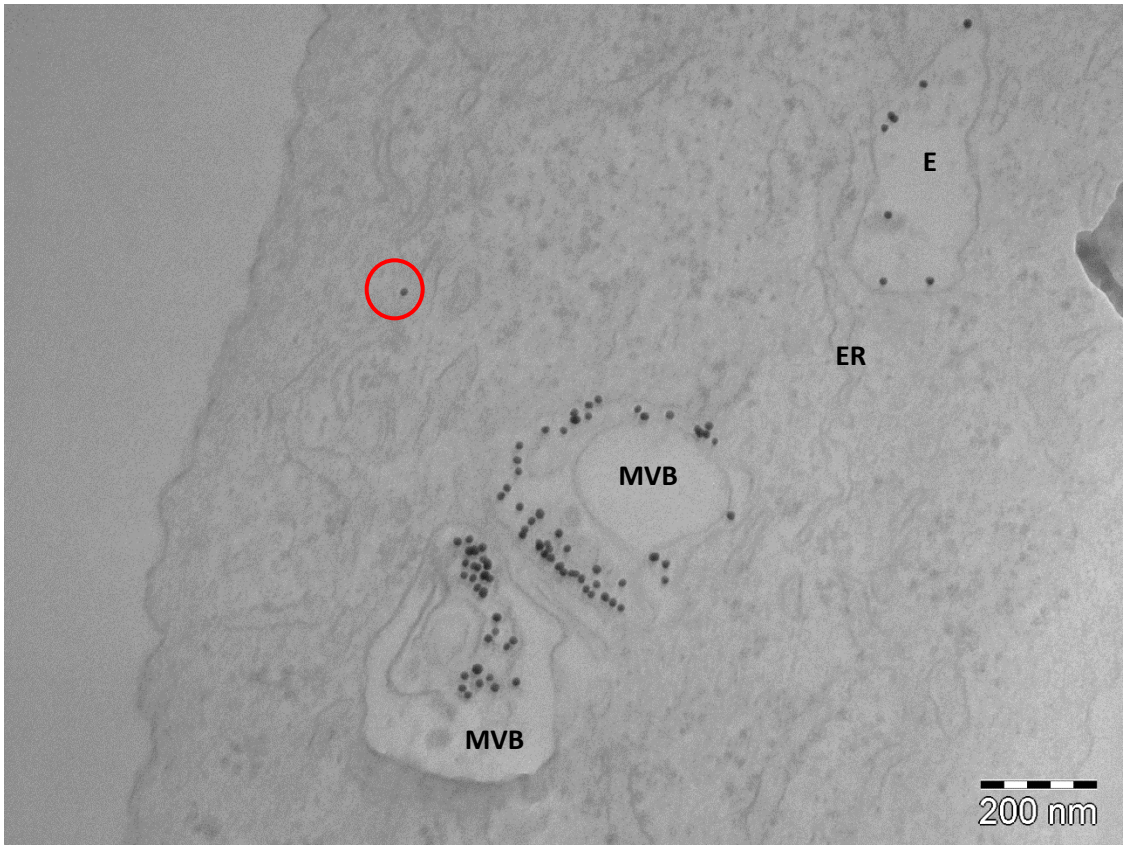

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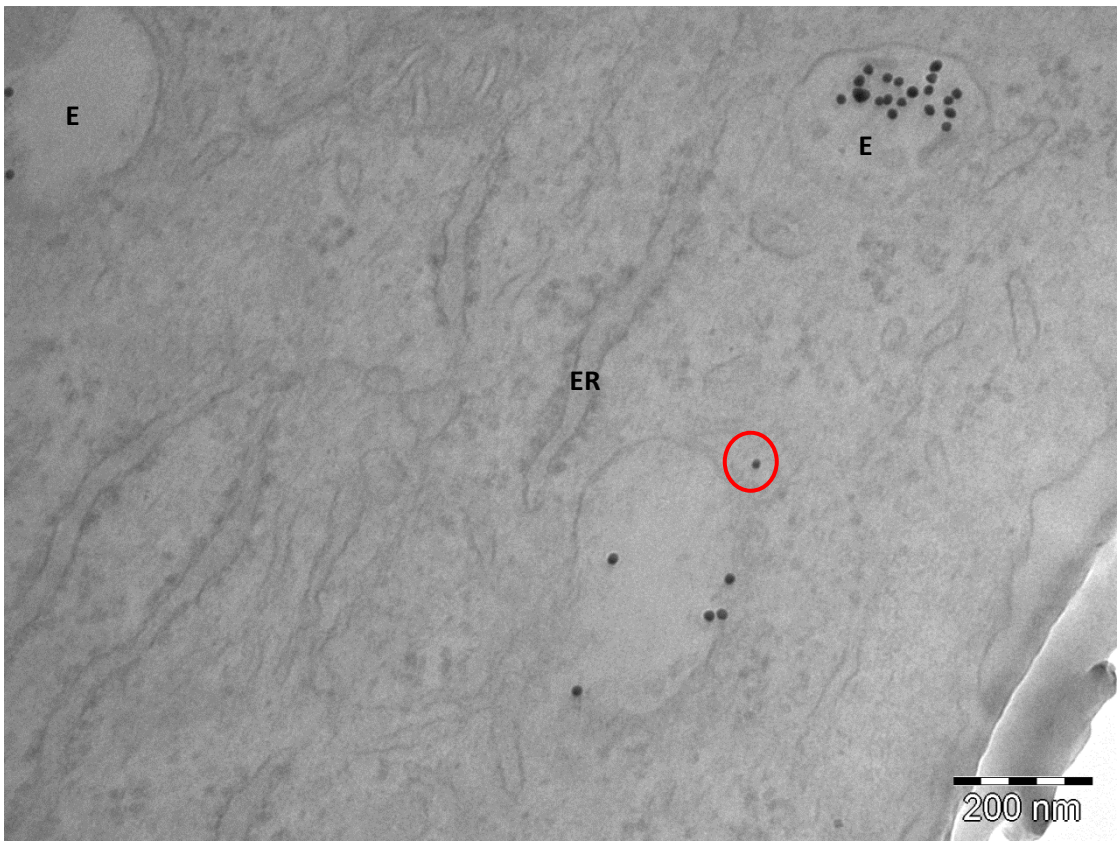

i

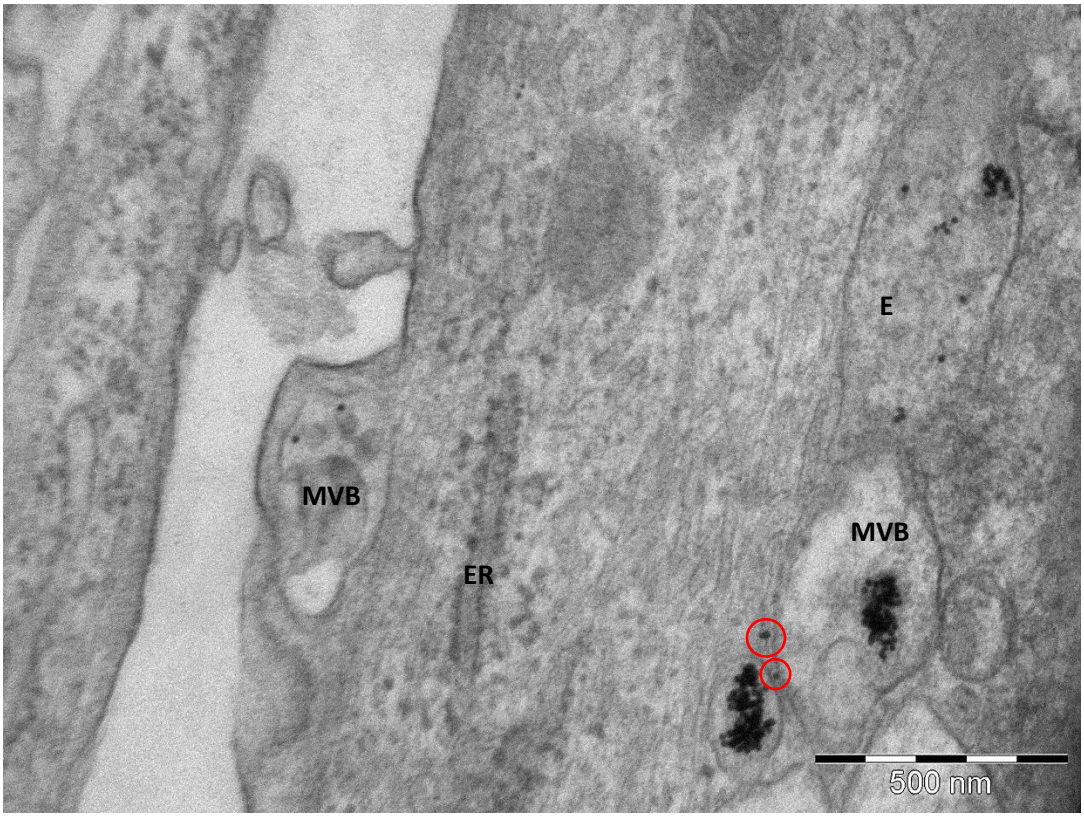

Figure S9. TEM images of cells incubated with general mRNA nano-probes (a-h) or Vimentin nano-probes (i) for 18 h. (a-d) show sections of 16 HBE cells, images (e-i) show sections of MRC-5 cells. **ER**: Endoplasmatic Reticulum, **E**: Endosome, **MVB**: Multivesicular Body, **Nu**: Nucleus.

S-VII References

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