Characterising antibodies targeting antisense oligonucleotide (ASO) modifications for quantification of intracellular trafficking and *in vivo* biodistribution

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1. Introduction

The efficacy of a new nucleic acid therapeutics (NATs) such as antisense oligonucleotides (ASO)s and small interfering RNAs (siRNA)s relies on multiple stages of extra- and intracellular trafficking. Thus, with the inherent vulnerability of nucleic acids and the necessity to escape the endosomal system for nuclear or cytoplasmic delivery, only a very small proportion of the intracellular NAT is estimated to be productive.

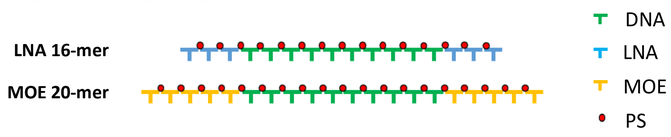
Assessing the uptake and efficacy of NATs often relies on fluorescent tagging to provide detectable signals for microscopy. As such, this approach is amenable for live imaging, although the exogenous tag undoubtedly influences the stability and kinetics of intracellular transport and does not represent the compound that is used clinically.

Therefore, better, quantitative methods of endogenous cellular and tissue distribution are needed in the field. In this study, we aim to validate new immunofluorescence staining reagents that target the modified oligonucleotide backbone for visualising ASOs both *in vitro* and *in vivo*. Using ModDetect reagents from Rockland Immunochemicals we have tested ASOs in a range of cell lines, cell model systems and mouse tissue by immunofluorescence.

Antibodies raised against phosophorothioate (PS) bonds successfully detect 2'MOE and LNA ASO gapmers, facilitating co-localisation studies with endolysosomal markers in cells and biodistribution in mouse tissue. Targeting the PS bond for NAT detection is irrespective of nucleotide sequence, rendering the system amenable for multiple target types.

2. Methods

ASO sequences: ASO gapmers targeting human (LNA) and mouse (2'MOE) *MALAT-1* / *Malat-1* were synthesised and QC'd at NATA as below:



Immunocytochemistry (ICC): Cells were stained with Hoechst 33342 for 5-10 mins, followed by Fixation using 4% PFA in PBS with 15-20 mins incubation; Blocking with 5% goat serum in 0.1% PBS-Tween (PBS-T); Primary antibody staining was carried out using anti-mouse PS03 (Rockland), diluted 1:1000 in PBS-T and incubated for 2hr room temperature. The signal was detected using Superclonal goat anti-mouse AF488 secondary (Invitrogen), diluted in PBS-T.

***In vivo* delivery and tissue processing:** ASOs were delivered SC at 50 mg/kg in wild-type C57BL6/J mice. After 48 hours, tissue was harvested, immersion-fixed in 10% NBF for 30 hours and processed in wax blocks. 8 μM sections were cut and mounted onto True-Bond slides.

Immunohistochemistry (IHC): Sections were de-waxed and rehydrated before blocking and staining using the M.O.M Immunodetection Kit (VectorLabs), with its standard protocol in combination with PS03 primary antibody (Rockland), diluted 1:2000. Mounting media with DAPI was used.

Quantitative (q)PCR: SYBR green qPCR was carried out for *MALAT-1* from a range of cell lines using *HPRT* as the housekeeping control gene.

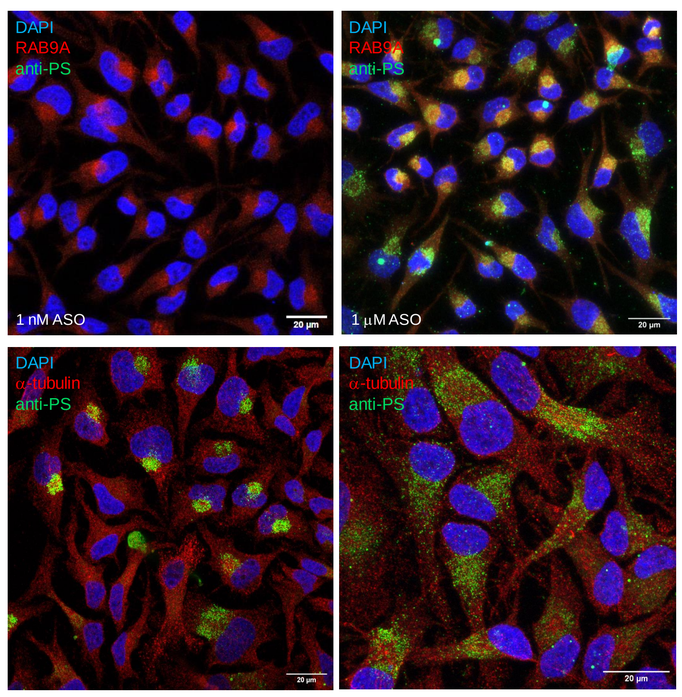
3. Detection sensitivity by ICC

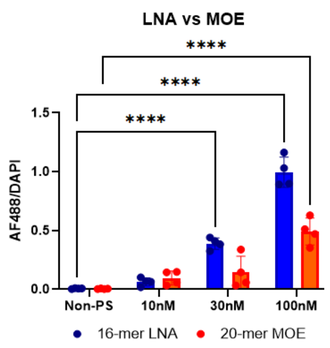
To test initially the sensitivity of the anti-PS antibodies, HeLa cells were dosed with the 16-mer LNA gapmer for 72 hours, either without (gymnosis) or with transfection reagent. Cells were immunostained with ModDetect clone PS03 (using a 488 secondary antibody) and the late endosomal marker RAB9A (594).

ASO doses as low as 1nM could be detected in transfected cells, with clustering of the signal adjacent to the nucleus reminiscent of the late endolysosomal compartments in HeLa cells.

No signal could be detected at 1nM after gymnotic delivery or using a non-PS containing 16-mer gapmer (data not shown).

The anti-PS signal intensity, normalised to DAPI nuclear staining, was quantified in SH-SY5Y cells transfected for 96 hours with the 16-mer LNA or the 20-mer 2'MOE gapmer. Both ASOs were detectable, with increasing mean normalised signal with higher dose levels.



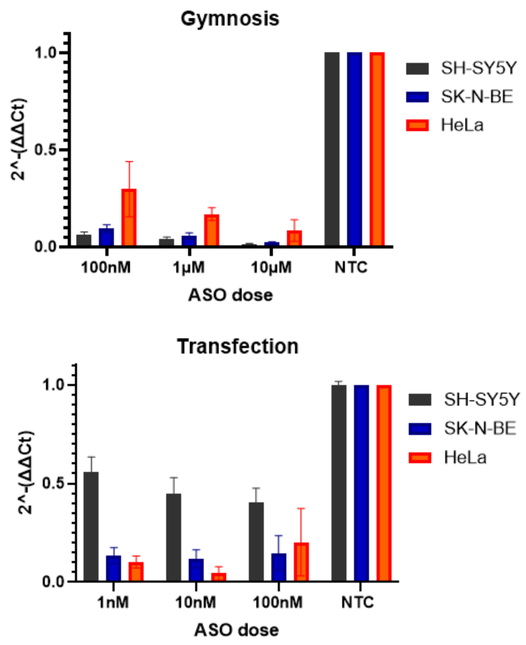


4. On-target efficacy across three cell lines

Anti-PS antibodies were tested across four different cell lines: SH-SY5Y, SK-N-BE, HeLa and H1299. Positive staining for the LNA gapmer ASO was observed in all lines (not shown).

To compare the immunocytochemical detection with on-target knockdown efficacy of *MALAT-1*, qPCR was performed across three different cell lines at different dose levels using either gymnosis or transfection. Data were normalised to a non-targeting control ASO (NTC).

By gymnosis, knockdown levels increased with increasing ASO dose. Interestingly, using transfection, increasing the dose to 100nM did not significantly enhance efficacy.

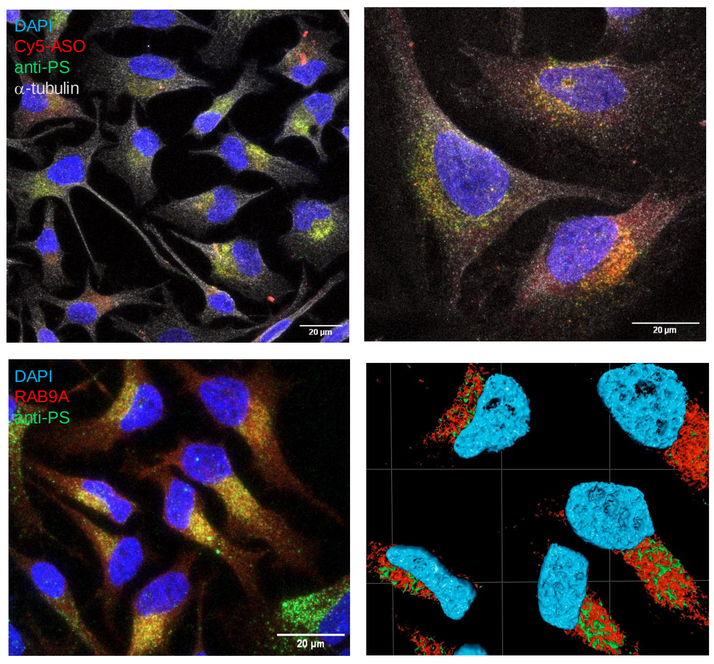


5. ICC colocalisation studies

A fluorescently labelled Cy5-ASO was used to perform a comparison with detection using anti-PS antibodies (top panels). Some regions of co-localisation (yellow) can be observed.

In addition, HeLa cells were co-stained with endosomal markers (e.g. RAB9A shown) to determine co-localisation with an un-labelled LNA ASO (lower panels).

Isosurface images, constructed by imaging the cells through a Z-stack reveal further details of potential colocalisation in the late endosome.



6. *In vivo* biodistribution

Mouse liver tissue was tested for detection of the 2'MOE gapmer 48 hours after systemic delivery.

Using the anti-PS PS03 antibody, signal could be detected consistently in the sinusoidal cells of the liver; strong signal in hepatocytes was not expected using an unconjugated gapmer.

7. Conclusions

- Both LNA and 2'MOE modified ASOs can be detected using ModDetect anti-PS antibodies by immunocytochemistry.
- Co-localisation studies suggest endosomal accumulation of ASOs after delivery by gymnosis or transfection in a range of cell lines and doses.
- *In vivo* detection of a 2'MOE ASO was successful in mouse liver tissue after systemic delivery

8. Acknowledgements

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