RIBOPRO

APPLICATION NOTE 02

Fluorescently Labelled mRNA for Dual-Channel Super-Resolution Microscopy

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INTRODUCTION

For cellular delivery in biomolecular research and clinical applications alike, mRNA is packaged into nanometer-sized nanoparticles using lipid formulations, peptides, or polymers. So far, only a few delivery approaches have yielded efficient delivery. To understand, how formulation parameters affect mRNA packaging, interaction with biological media and cellular uptake, ideally analytical approaches should be used that enable an investigation of mRNA nanoparticles at the relevant spatial dimensions which means below the resolution limit obtained by conventional light microscopies.

STORM (stochastic optical resolution microscopy) super-resolution microscopy provides the means to visualize individual mRNA nanoparticles and even determine the stoichiometry of molecules within these nanoparticles.[1] A prerequisite for conducting STORM analyses is the labeling of the molecules of interest with photoswitchable fluorophores. Here, we used mRNA labelled with AZDye488 and AZDye647 to simultaneously analyze two different types of mRNA nanoparticles. In this way, we were able to show that these two particle types neither aggregate nor mutually exchange mRNA molecules.

METHODS

AZDye488- and AZDye647-labelled mRNA (RIBOPRO) were formulated with two different cationic peptides (peptide 1 and peptide 2) by a stream method as described previously. In short, aqueous solutions of both, peptide and mRNA were dispensed simultaneously from electronically controlled pipettes with both pipette tips held in contact through a custom-made mold.[2]

The AZDye488-mRNA coded for secreted nanoluciferase (SecNLuc), thus had a length of 638 nucleotides, the AZDye647-mRNA coded for eGFP and had a

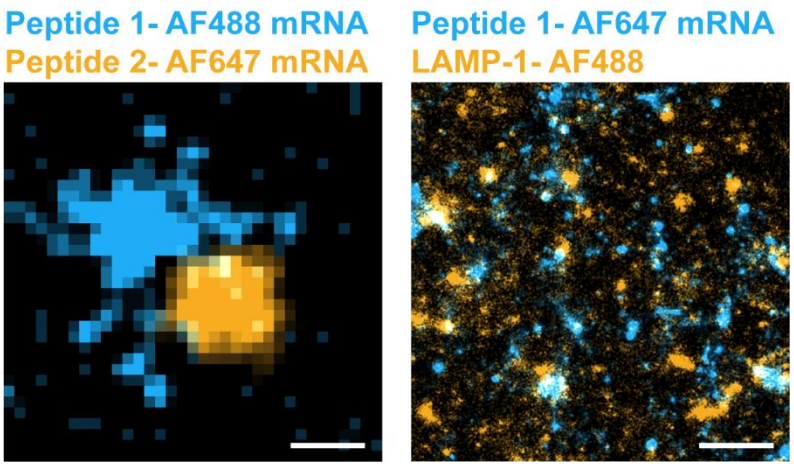
length of 758 nucleotides (excluding poly A-tail). A total of 25% of all UTPs were fluorescently labelled, resulting in labeling degree of 5.4% and, 3.2% respectively (calculated excluding poly A-tail).

STORM analyses were performed with a dual channel STORM microscope using a custom-built low-drift inverted microscope setup. In the excitation path, three laser light sources were spatially overlapped: a 1000mW 639nm laser, a 500mW 488nm laser (Genesis MX STM OPSLaser System, Coherent), and a 100mW 405nm laser (OBIS LX Laser System, Coherent). These lasers were focused at the back focal plane of the imaging objective (APON 60XOTIRF /1.49 OIL, Olympus America Inc.) and emitted light was recorded with a scientific CMOS camera (ORCA-Flash4.0 V2, Hamamatsu). For dual-colour imaging, the light was filtered using a triple band dichroic mirror (ZT405/488/635rpc, Chroma) in combination with an emission filter (FF01-565/133-25, Semrock and/or ET655lp, Chroma). The entire setup was controlled using custom-written software in Matlab (MathWorks, Massachussets, USA)

Samples were dispensed on coverslips and imaged in about 1ml of OxEA buffer.[3] Typically, a total of 50,000 frames was acquired within a region of interest (ROI) of 300×300 pixels for each channel. The pixel size was 111 nm, and an exposure time of 10 ms was used.

Images were reconstructed from 10-50x10^6 fit positions, with a detection threshold of 100 and 200 photons for the 488 nm and 639 nm channels, respectively. Typical uncertainty mode values were 12nm for the 639 nm channel and 15nm for the 488 nm channel. Subsequently, images were reconstructed using the average shifted histograms method, with a rendering pixel size of 10nm.

For analysis of cellular uptake and colocalization with lysosomes, Hela cells were incubated with particles incorporating AZDye647-mRNA for 1 hour followed by fixation, permeabilization and indirect immunofluorescence for the lysosomal marker LAMP1 using an AZDye488-labelled secondary antibody.



Dual-colour super-resolution microscopy of peptide-mRNA nanoparticles deposited on glass. (A) Average shifted histogram intensity projections of fluorescently-labelled mRNA nanoparticles. Scale bar represent 50 nm. (B) Fluorescently-labelled mRNA nanoparticles colocalize with the endolysosomal compartments as evidenced by LAMP-1 and AZ647-mRNA co-occurrence. Scalebars represent 1 µm.

RESULTS

Incorporation of labelled mRNAs yielded nanoparticles that were only slightly smaller than the ones formulated with unlabelled mRNA as determined by dynamic light scattering, thus demonstrating that the labels did not interfere with RNA packaging. Both types of nanoparticles yielded high-quality super-resolution images with on average 8,000 detections per particle for 50,000 frames for the 488 channel and 20,000 detections per particle for 50,000 frames for the 647 channel. AZDye647 gave a higher photon yield than the 488 channel.

The median particle diameters as derived from an image analysis of clustered photon events were 98.91± 21.8 for Peptide 1-AZ488 and 96.82 ± 21.6 nm for Peptid-AZ647, thus very similar to the 64.11 ± 0.73, and 68.38 ± 0.31 nm measured by DLS, respectively. Importantly, as derived from super-resolution images there was neither aggregation of nanoparticles, nor exchange of mRNA.

Cells showed uptake of nanoparticles and colocalization with endosomes.

CONCLUSIONS

AZDye-labelled mRNAs provide the brightness for super-resolution microscopy of nanoparticle dimensions and cellular uptake. The ability to incorporate different fluorophores provides versatility for dual channel applications.

RIBOPRO PRODUCTS

Fluorescently labelled SecNLuc mRNA

mRNA properties	
Sequence length	638nt (excluding poly A-tail)
UTRs	RIBOPROs 3' and 5' UTR
5' end	Cap1
Poly A-tail length	150nt
Fluorescent label	AZDye 488
% of labelling	5,4% (excluding poly A-tail)

Fluorescently labelled eGFP mRNA

mRNA properties	
Sequence length	758nt (excluding poly A-tail)
UTRs	RIBOPROs 3' and 5' UTR
5' end	Cap1
Poly A-tail length	150nt
Fluorescent label	AZDye 647
% of labelling	3,2% (excluding poly A-tail)

REFERENCES

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