Counting mRNAs:
The single-molecule in vitro transcription (smIVT) assay

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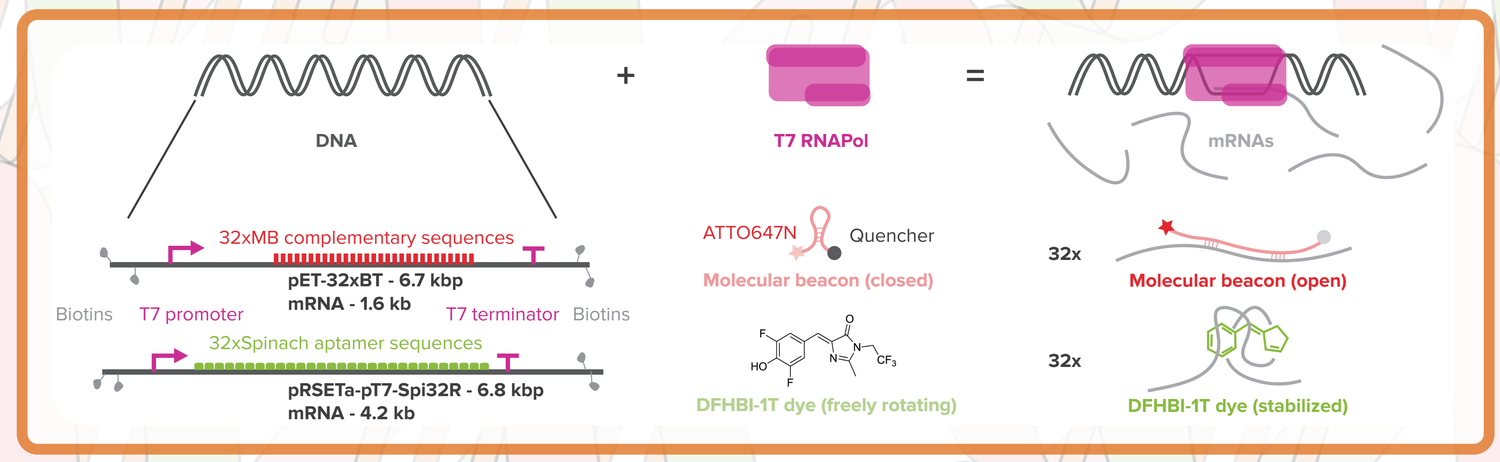
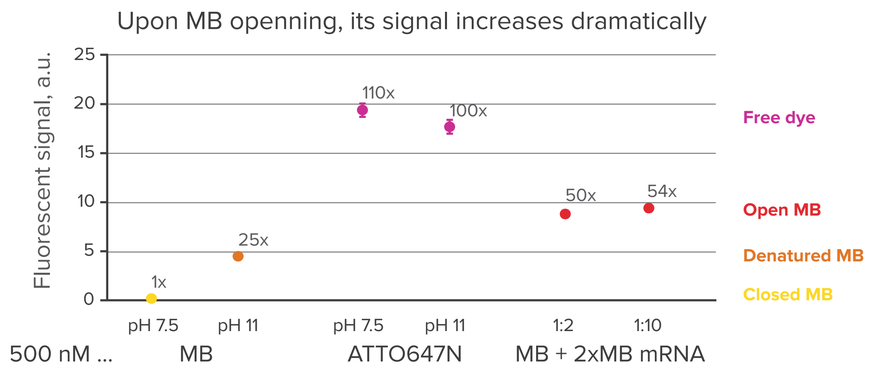
With the smIVT assay, we aim to provide the SynCell community with a single-molecule imaging tool to observe mRNA synthesis in real-time, compare different commercially available or lab-made transcription systems and study transcription at a single DNA per synthetic cell concentration.

Here we utilize two approaches. Both are reliant on fluorescent probes that become more fluorescently active after interactions with the transcript mRNA: molecular beacons (MB) with ATTO647N dye and Spinach aptamers. In both cases we use 32 repeats in order to increase signal to noise ratio and resolve individual mRNAs as they appear.

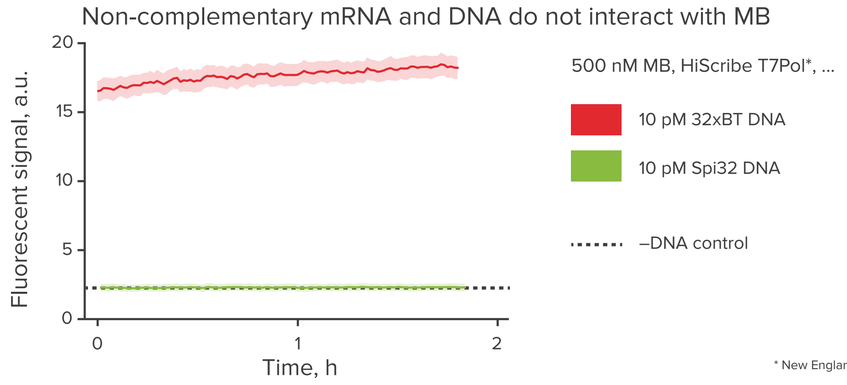


Molecular beacon is a lighting-up sequence-specific probe

Upon MB openning, its signal increases dramatically



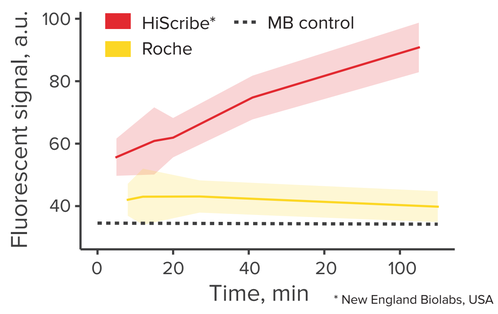
Non-complementary mRNA and DNA do not interact with MB



* New England Biolabs, USA

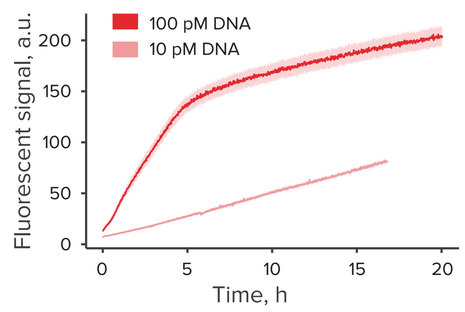
Multiple MB enable single-molecule mRNA detection

HiScribe T7RNAPol* outperformed others and was used for further experiments

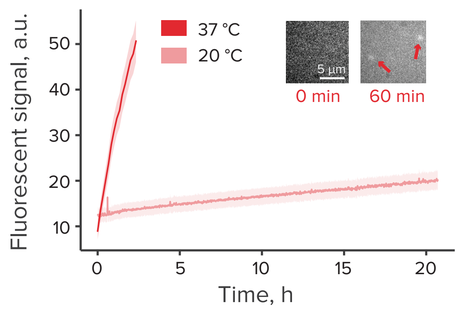


* New England Biolabs, USA

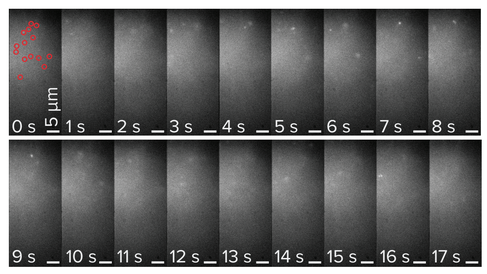
Higher DNA leads to faster mRNA production



Reaction runs optimally at 37 °C and produces individually-resolved mRNAs



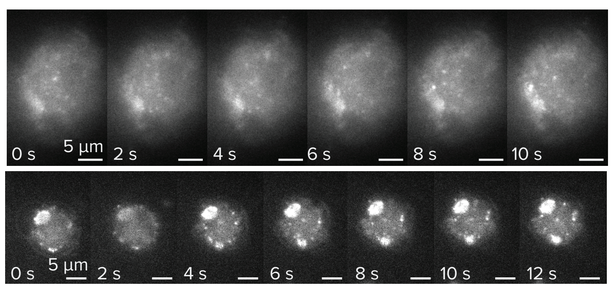
Individual 32xMB mRNAs can be tracked



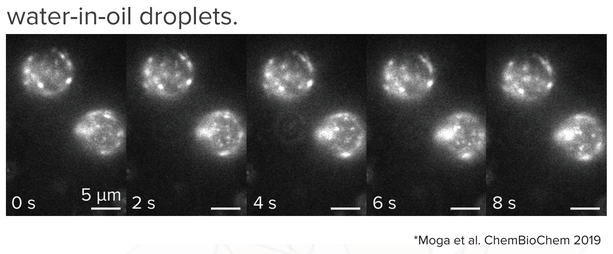
500 nM ATTO647N-MB, 10 pM DNA, 50 U T7 RNA Polymerase, 10 mM each NTP
40 mM Tris-HCl, 19 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine
30 U/ml unorganic pyrophosphase (yeast), 12,000 U/ml ribonuclease inhibitor

Single DNA and mRNA molecules could be imaged inside giant unilamellar vesicles (GUVs) for a confined transcription reaction

Transcription reaction solution was incubated for 1 h at 37 °C. It was then encaspsulated inside GUVs using inverted emulsion method* and imaged.

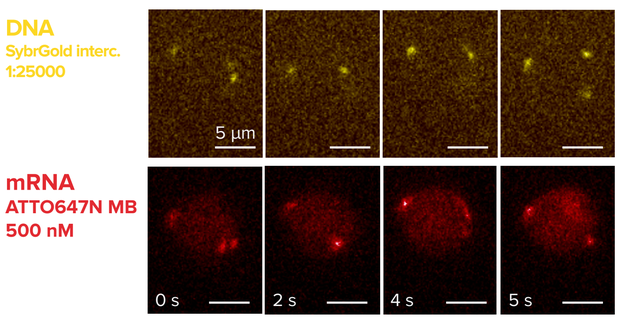


Individual mRNA molecules were also imaged in water-in-oil droplets.



*Moga et al. ChemBioChem 2019

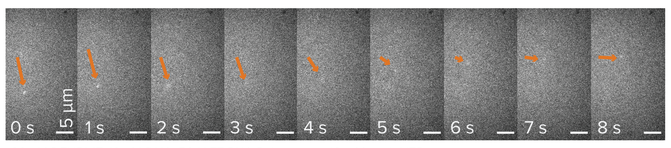
Finally, transcription mixture was encapsulated inside of GUVs and placed at 37 °C. An aliquot was taken after 1 hour to observe DNA and mRNA molecules inside of the vesicles. We have successfully detected both. Although, the yield of GUV production was lower and there were membrane imperfections, which seemed to accumulate MB, DNA and intercalator



500 nM ATTO647N-MB, 10 pM DNA, 50 U T7 RNA Polymerase, 10 mM each NTP, 1:25000 SybrGold
40 mM Tris-HCl, 19 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 200 mM sucrose
30 U/ml unorganic pyrophosphase (yeast), 12,000 U/ml ribonuclease inhibitor

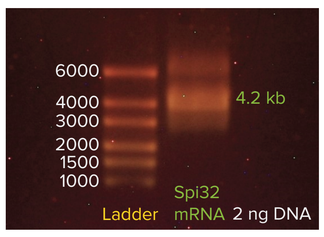
Multiple Spinach aptamers are a worse alternative to multiple molecular beacons

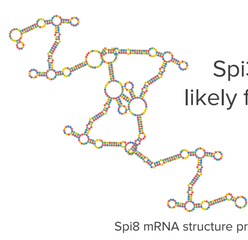
Individual Spi32 mRNAs can be tracked continiously in solution. However, compared to ATTO647N, DFHBI-1T dye bleaches faster and has lower signal enchancement upon binding, which leads to more background noise and worse signal-to-noise ratio.



20 uM DFHBI-1T, 10 pM DNA, 50 U T7 RNA Polymerase, 10 mM each NTP
40 mM Tris-HCl, 19 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine
30 U/ml unorganic pyrophosphase (yeast), 12,000 U/ml ribonuclease inhibitor

32x Spinach sequences likely fold on themselves. Only a few bright spots could be observed in 10 pM DNA transcription reaction mix after 1 h at 37 °C. At the same time, Spi32 mRNA imaged on the gel suggests abundant full-length mRNA were produced.





Spi32 sequence likely folds on itself

Spi8 mRNA structure predicted by EternaFold

What's next for smIVT project?

Utilizing multiple molecular beacons has been shown here to be a prospective approach to visualize single mRNAs produced by T7 polymerase. It was demonstrated at pM DNA concentrations similar to that of living cells. Our next goal is to improve the localization of this reaction through encapsulation of the mix in GUVs while measuring continuously on a heated microscope stage, thus keeping the transcription running.

Another step towards a reliable and quantitative transcription assay will be calculating the reaction rate from the number of produced mRNAs and the initially available DNA templates. For the latter, we aim to count individual DNA molecules using fluorescently labeled steptavidin that will not impede T7 processivity.

Finally, transcription is only half of the story. Including translation of the fluorescent proteins will be another significant milestone. After all, our ultimate goal in this project is to find the optimal cell-free expression systems and conditions while examining TxTl coupling and noise effects.

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