Christopher D. Reinkemeier[1,2,3*], Gemma Estrada Girona[3], Edward A. Lemke[1,2,3]
1 Biocentre, Departments of Biology and Chemistry, Pharmacy and Geosciences, Johannes Gutenberg-University Mainz (Germany); 2 Institute of Molecular Biology (Mainz, Germany);
3 Structural and Computational Biology Unit & Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, (Heidelberg, Germany)
* Christopher.Reinkemeier@embl.de

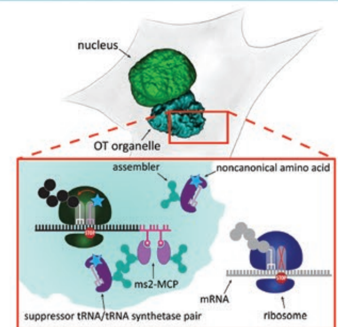
EMBL

Designer membraneless organelles enable orthogonal translation in eukaryotes

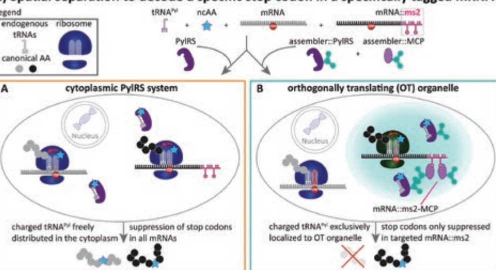
JGU JOHANNES GUTENBERG UNIVERSITÄT MAINZ

imb Institute of Molecular Biology

Abstract:
Genetic code expansion (GCE) is a powerful tool to study, tailor and control protein function with single-residue precision[1,2]. It is widely used to perform labeling for microscopy or to photocontrol proteins. This is achieved by introducing an orthogonal tRNA/synthetase suppressor pair into the host, to recode a stop codon to incorporate a noncanonical amino acid (ncAA) into the nascent chain. This technique is codon-specific, but it cannot select specific mRNAs, so naturally occurring stop codons could be suppressed leading to potential interference with housekeeping translation.
Nature avoids cross-talk between cellular processes by confining specific functions into organelles. We aimed to design an organelle dedicated to protein engineering, but as translation is a complex process requiring hundreds of factors to work together, membrane-encapsulation would not be feasible. Inspired by the concept of phase separation we hypothesized that such an organelle could instead be designed membraneless.
Phase separation can generate high local concentrations of proteins and RNAs in cells and has recently gained attention owing to its role in the formation of specialized organelles such as nucleoli or stress granules. Despite being membraneless and constantly exchanging with the cytoplasm/nucleoplasm, these organelles still perform complex tasks, such as transcription.
We combined phase separating proteins[3-5] with microtubule motor proteins[6] to generated orthogonally translating organelles in living cells that contain an RNA-targeting system[7], the stop codon suppression machinery and ribosomes. These large organelles enable site- and mRNA-specific ncAA incorporation, decoding one specific codon exclusively in the mRNA of choice.
Our results demonstrate a simple yet effective approach to the generation of semi-synthetic eukaryotic cells containing artificial organelles to harbor two distinct genetic codes, providing a route towards customized orthogonal translation and protein engineering.



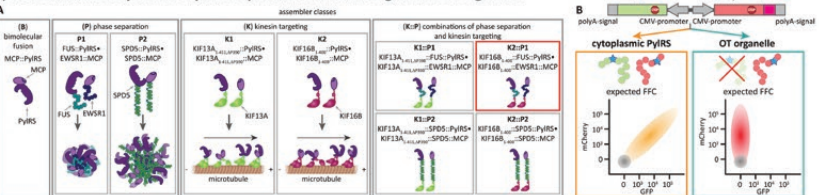
1) Spatial separation to decode a specific stop codon in a specifically tagged mRNA

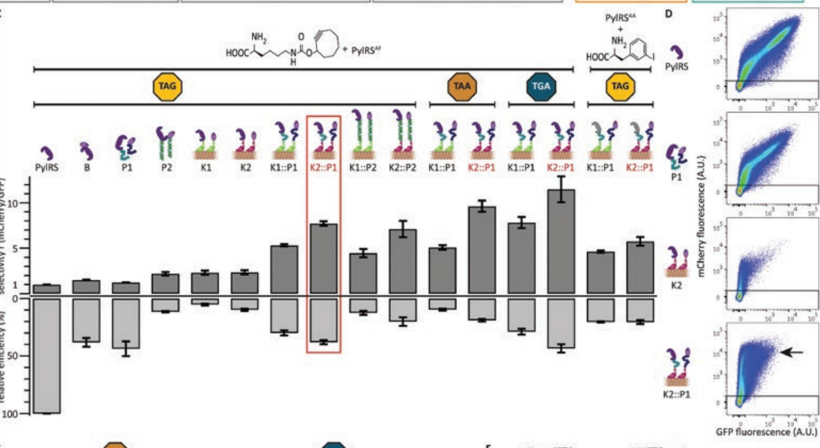


Hypothesis: We hypothesized that it is possible to create an orthogonal translation system by spatially enriching specific components of the GCE machinery in an artificial orthogonally translating (OT) organelle.

(A) The cytoplasmic pyrrolysyl aminoacyl-tRNA-synthetase (PylRS) aminoacylates its cognate stop codon suppressor $tRNA^{Pyl}$ with an ncAA[8,9,10]. This leads to cotranslational ncAA incorporation when the respective stop codon occurs in the mRNA of the POI. However, many endogenous mRNAs terminate on the same stop codon, potentially leading to ncAA misincorporation.
(B) Spatially enriching components for GCE, i.e. the mRNA of the POI, PylRS, $tRNA^{Pyl}$, and ribosomes through the use of "assemblers" to an OT organelle should enable selective translation of only the recruited mRNAs. Whereas, in (A) GCE is stop codon specific, in (B), it is stop codon- and mRNA-specific.

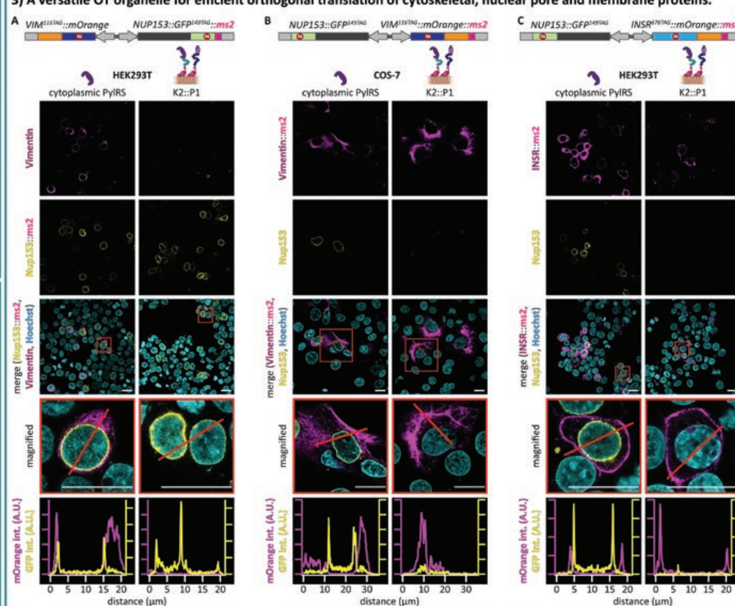
2) Local enrichment by means of phase separation is a means to generate OT organelles





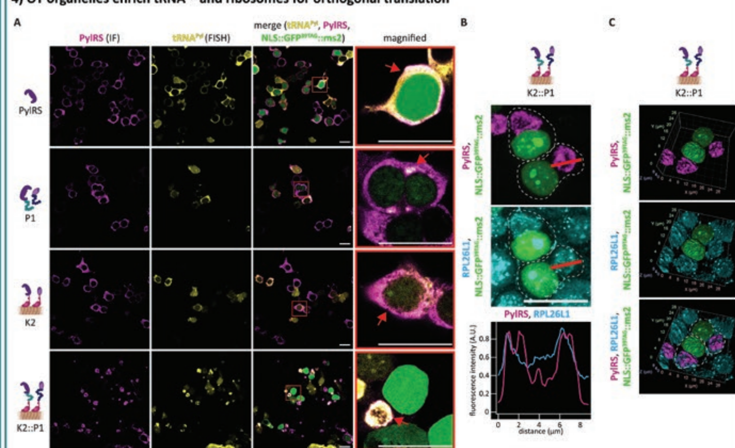
(A) Schematic overview of assemblers to create OT organelles.
(B) A dualcolor reporter to evaluate OT organelle performance. GFP and mCherry mRNA, containing stop codons at permissive sites, are expressed from one plasmid, ensuring a constant mRNA ratio throughout experiments. Cytoplasmic PylRS yields production of GFP and mCherry [orange diagonal in fluorescence flow cytometry (FFC)]. The OT organelles enable selective stop codon suppression of mCherry::ms2 mRNA, resulting in an mCherry positive and GFP negative population (schematically drawn in red).
(C) Bar graph analysis of OT organelles. The indicated systems were coexpressed with $tRNA^{Pyl}$ and the dualcolor reporter (GFP^{39STOP}, $mCherry^{185STOP}$::ms2). GCE was performed in presence of the indicated ncAAs, and cells were analyzed by FFC. Dark gray bars represent releative selectivity r (mean fluorescence intensities of mCherry/GFP normalized to cytoplasmic PylRS). Light gray bars show relative efficiency (mean mCherry fluorescence intensity normalized to cytoplasmic PylRS). Shown are the mean values of at least three independent experiments, error bars represent the SEM.
(D) FFC dotplots corresponding to selected systems from (C), as indicated by the symbols.
(E) FFC dotplots showing efficient and selective translation of Ochre and Opal codons by the $OT^{K2::P1}$ organelle.
(F) FFC dotplots showing, that swapping the ms2-loops from mCherry to GFP also inverts the selectivity of the OT organelles.

3) A versatile OT organelle for efficient orthogonal translation of cytoskeletal, nuclear pore and membrane proteins.



(A-C) Confocal images of cells expressing selected proteins using PylRS (left columns) or the $OT^{K2::P1}$ organelle (right columns). ($tRNA^{Pyl}$ and SCO present in all experiments)
(A) HEK293T cells transfected with VIM^{116TAG}::mOrange & $NUP153$::GFP^{149TAG}::ms2 selectively express tagged Nup153::GFP^{149TAG}::ms2 (yellow) with the $OT^{K2::P1}$ organelle, and no $Vimentin^{116TAG}$::mOrange (magenta).
(B) COS-7 cells transfected with $NUP153$::GFP^{149TAG} & VIM^{116TAG}::mOrange::ms2 exclusively express tagged Vimentin::mOrange::ms2 (magenta) using the $OT^{K2::P1}$ organelle.
(C) Using the $OT^{K2::P1}$ organelle HEK293T cells can even selectively express the membrane protein $INSR^{676TAG}$::mOrange::ms2 (magenta), while not expressing the cotransfected $NUP153$::GFP^{149TAG}. This highlights, that the $OT^{K2::P1}$ can even participate in the complex translation of membrane proteins, for which the ribosome needs to interact with the ER.

4) OT organelles enrich $tRNA^{Pyl}$ and ribosomes for orthogonal translation



(A) IF and FISH imaging of HEK293T cells expressing $tRNA^{Pyl}$ and the indicated systems. Green nuclei report on successful Amber suppression. Assembler::PylRS (magenta) fusions nicely colocalize with $tRNA^{Pyl}$ (yellow). Scale bars, 20 µm.
(B) Maximum intensity Z-projection of IF image Z-stacks of HEK293T cells expressing the $OT^{K2::P1}$ organelle. Ribsomes colocalize with the OT organelle. [PylRS (magenta), RPL26L1 (cyan), NLS::GFP^{39TAG}::ms2 (green), line profiles for PylRS and RPL26L1 (red line, magenta and cyan curves)]. Scale bars, 20 µm.
(C) 3D reconstructions corresponding to B [PylRS (magenta), RPL26L1 (cyan), NLS::GFP^{39TAG}::ms2 (green)]

5) Designer membraneless organelles equip cells with a second genetic code to orthogonally translate selected mRNAs[11]

in cell organelle formation

mRNA::ms2 and $tRNA^{Pyl}$ recruitment

ribosome recruitment

orthogonal translation by the $OT^{K2::P1}$ organelle

KIF16B::FUS::PylRS and KIF16B::EWSR1::MCP form a synthetic organelle inside a living cell. PylRS recruits $tRNA^{Pyl}$, depleting it from the cytoplasm, while MCP recruits mRNA::ms2. Ribosomes and translation factors can also enter the organelle. As $tRNA^{Pyl}$ is only available in the organelle only the recruited mRNA can be translated with an expanded genetic code by a spatially distinct set of ribosomes. Meanwhile, mRNAs that are not recruited to the OT organelle are subject to normal translational processing of the host machinery, so that a stop codons will terminate translation.

References
1) C. C. Liu, P. G. Schultz, Adding new chemistries to the genetic code. Annu. Rev. Biochem. (2010)
2) J. W. Chin, Expanding and reprogramming the genetic code. Nature (2017)
3) M. Altmeyer et al., Liquid demixing of intrinsically disordered proteins is seeded by poly(ADP-ribose). Nat. Commun. (2015)
4) A. Patel et al., A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. Cell (2015)
5) J. B. Woodruff et al., The centrosome is a selective condensate that nucleates microtubules by concentrating tubulin. Cell (2017)
6) V. Soppina et al., Dimerization of mammalian kinesin-3 motors results in superprocessive motion. Proc. Natl. Acad. Sci. U.S.A. (2014)
7) E. Bertrand et al., Localization of ASH1 mRNA particles in living yeast. Mol. Cell (1998)
8) I. Nikić et al., Debugging eukaryotic genetic code expansion for site-specific click-PAINT super-resolution microscopy. Angew. Chem. (2016)
9) T. Plass et al., Genetically encoded copper-free click chemistry. Angew. Chem. (2011)
10) Y. S. Wang et al., Genetic incorporation of twelve metasubstituted phenylalanine derivatives using a single pyrrolysyl-tRNA synthetase mutant. ACS Chem. Biol. (2013)
11) C. D. Reinkemeier, G. Estrada Girona, E. A. Lemke, Designer membraneless organelles enable codon reassignment of selected mRNAs in eukaryotes. Science (2019)

Acknowledgements: We thank I. Schneider as well all members of the Lemke laboratory in particular C. Koehler and J. Caria for helpful discussions. We thank the EMBL flow cytometry core facility and the advanced light microscopy facility for expert assistance. Funding: The Lemke laboratory acknowledges generous support from European Research Council (ERC) SMPFv2.0, SPP1623, and SFB1129 and the Gutenberg Research College.