TRAPS - A modular condensate-based RNA *in vivo* sensory platform

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ABSTRACT

RNA is an essential biomolecule, with both functional and structural versatility. Understanding RNA dynamics, localization, and expression in living cells is therefore essential for decoding cellular function. Current RNA imaging methods often involve cell fixation or extensive genetic modifications and are limited in detecting low RNA concentrations. Here, we present RNA detection through Targeted RNA Activated Phase Separation (TRAPS). TRAPS is a novel, modular method for in vivo RNA detection using catalytically inactive Cas13 fused to GFP-tagged scaffold domains. With TRAPS, fluorescent condensates form upon multivalent binding of the target RNA by Cas13, allowing easy detection. This enables real-time detection of endogenous RNAs, advancing the toolbox to detect RNA in lower abundance and shorter lifetime. As a proof-ofconcept experiment, we target mCherry RNA in S. cerevisiae. The Cas13 component of TRAPS offers easy adaptation to different target RNAs and varying in vivo conditions, making it a versatile method to investigate a range of biological processes, such as stress response, tissue morphogenesis, and progression of disease.

Index Terms - RNA, Detection, Condensation, Phase Separation

I. INTRODUCTION

For the understanding of cellular function, it is crucial to comprehend the dynamics, localization, and expression patterns of RNA molecules within living cells. The roles RNA has in a cell reach far beyond its original discovery as just an intermediate molecule between DNA and protein. The earliest discoveries hinting at this were the existence of messenger RNA (mRNA), transcribed from only a small fraction of the full genome and responsible for translation (Brenner et al., 1961; Gros et al., 1961). Since then, non-coding RNA (ncRNA) has been found in a variety of forms with catalytic activity, i.e. ribozymes, or capable of regulating gene expression, i.e. small interfering (siRNA) and microRNA (miRNA) (Fu, 2014; Strobel & Cochrane, 2007). Unsurprisingly, the versatility of RNA form and function leads RNA to play a role in most cellular processes. Consequently, RNA research has been and will continue to becrucial for a better understanding of biological processes such as development, stress response and more (Muñoz-Velasco et al., o. J.). In addition, RNA research has led a new avenue of therapeutics, diagnostics, and medicine with notably the mRNA-based vaccines during the global COVID pandemic (Barta & Jantsch, 2017; Chatterjee et al., 2023; Zhang et al., 2023). This makes RNA one of the most diverse and influential classes of biomolecules with growing global research interest, highlighted by four Nobel Prizes linked to RNA since 1965. Simultaneously, RNA detection methods have been developed and improved over the years to facilitate RNA research and elucidate its many functions in biology. In this paper, we will briefly describe the current RNA detection methods, their strengths and limitations, and propose a novel RNA detection method based on liquid-liquid phase separation (LLPS).

II. Common RNA Detection Methods

One of the most widely used RNA detection methods is realtime reverse transcription polymerase chain reaction (RT-PCR) (Afzal, 2020; Sullivan et al., 2023). Here, the cell is lysed and the RNA extracted, after which it is transcribed into cDNA with reverse transcriptase and amplified using PCR. Although RTPCR technique is very sensitive and provides fast results, it requires the cell to be lysed prior to analysis, making RT-PCR unsuitable for real-time expression studies (Afzal, 2020; Sullivan et al., 2023).

Another widely used technique is fluorescence in situ hybridisation (FISH). FISH utilizes fluorescently labelled oligonucleotides that hybridize with the targeted RNA (Singer & Ward, 1982). The presence and location of the target RNA in cells can then be observed using fluorescence microscopy. However, FISH generally requires heavy tissue preparation and fixing cells with formaldehyde

(Eltoum et al., 2001), limiting its applicability to study RNA dynamics *in vivo*. While both RT-PCR and FISH are limited in observing realtime transient RNA behaviour, several other systems were developed to address this limitation. These techniques often utilize oligonucleotides as molecular beacons (MB) (Cao et al., 2022). An MB is an oligonucleotide with an antisense stemloop that binds the target RNA. A fluorophore is added to one end of the loop and a quencher on the other end. The stem-loop ensures that the fluorophore and quencher remain in close proximity, suppressing fluorescence. The MB unfolds upon hybridization with the target RNA, separating the quencher and the fluorophore, resulting in a fluorescent signal (Marras, 2002). A limitation of MBs is the transport into the cells by protein carriers, making it prone to false positives due to nucleic acid degradation (Chen et al., 2007).

In recent years, novel *in vivo* RNA detection methods based on Cas13 have been developed to address some of these limitations. Cas13 can bind and cleave specific RNA sequences based on a highly modular guide RNA (Zhu et al., 2024). By mutating the catalytic center, RNA cleavage is deactivated (dCas13), resulting in a modular RNA binding platform. The dCas13-RNA binding can be visualized by fusing a fluorescent protein to the dCas13. Consequently, when target RNA is present, the localized fluorescence of dCas13 can be detected (Yang et al., 2019). This method is most effective at higher RNA concentrations, since a low amount of target RNA leads to only a fluorescence increase and a poor signal-to-noise ratio (SNR).

III. Improving signal strength by LLPS

In the previously discussed methods the signal was generated upon binding to the target RNA. The signal strength is therefore determined by the binding dynamics of the reporting probe to RNA, which following the law of mass action results in the fluorescent signal being linearly dependent on the concentration of the target. The lower the target's concentration, the lower the signal on average and more stochastic itself, making it harder to differentiate from background autofluorescence and measurement device noise. Liquid-liquid phase separation (LLPS) of biomolecules, like proteins and nuclear acid polymers leads to the formation of at least two coexisting phases with different compositions, usually appearing as droplets in a larger phase, and are used for a diverse set of cellular functions (Banani et al., 2017). The spontaneous mixing in multicomponent systems is driven by entropy, during phase separation an enthalpic contribution to the free energy, originating from molecular interactions can win over, causing the formation of separate phases.

Using the target molecule as a scaffold molecule, meaning it facilitates the interactions necessary for phase separation, its concentration determines when phase separation into droplets occurs and the size of the fluorescent droplets. This offers a less noise sensitive approach of concentration measurements compared to fluorescence intensity based methods. Below the critical concentration required for LLPS there is only one mixed phase and the SNR behaves as described for the previous methods. Therefore the critical concentration of phase separation needs to be optimized to be as low as possible.

Biological condensate systems tend to use multivalent scaffold molecules, as having many binding sites lowers the critical concentration and increases the critical temperature, allowing bio-molecules to phase separate easier (Banani et al., 2017). Mechanistically having multiple binding domains causes cooperative binding dynamics and local clustering of the target RNA and the probes, facilitating stronger interactions among each other at lower concentrations. It should also be noted that very strong specific interactions are commonly related to the assembly of networks, while weaker less specific interactions in greater numbers (high valency macromolecules and intrinsically disorder domains) are suggested to play a major role in condensates maintaining their liquid like properties (Banani et al., 2017).

While previous methods work best at high RNA concentrations, lower amounts of target RNA lead to a decrease in fluorescence and worse signal-to-noise ratio (SNR). We aim to improve upon current techniques through liquid-liquid phase separation (LLPS) in the presence of the target RNA conditionally forming fluorescent condensates. The formation of these condensates significantly increases the local fluorophore concentration, thereby allowing us to detect RNA at a lower concentration.

IV. Design of phase separating RNA binding sensory system

The proposed system is based on the scaffold system engineered by Heidenreich et al. (2020). Their work presents a modular, synthetic system designed to investigate phase separation in living cells from first principles. Crucial for controlling condensation in this system is the toxinantitoxin interaction with intermediate affinity. We adapted this scaffold system to the TRAPS system. The system is built on the same two protein interaction system, the toxin E9and the immunity protein Im2 and also the tetramerization domain, 1AIE.

However, we also added a dCas13, functioning as an RNA Binding Protein (RBP). The E9 is fused to the tetramerization domain and green fluorescent protein GFP, forming a fluorescent tetramer. The RBP is fused to the Im2, connecting it to the tetrameric unit (Figure 1. A). The toxin and antitoxin will constantly bind and unbind, allowing LLPS. There are different gRNAs to bind multiple RNA target sites. If the RNA of interest is present, it will bind to the RBP and start connecting different tetrameric units of the system. This process forms a network and ultimately leads

to phase separation (Figure 1.C). By forming the condensates, the GFP fluorescence gets concentrated. As mentioned before, we want to use these effects to passively sense the signal of the target RNA.

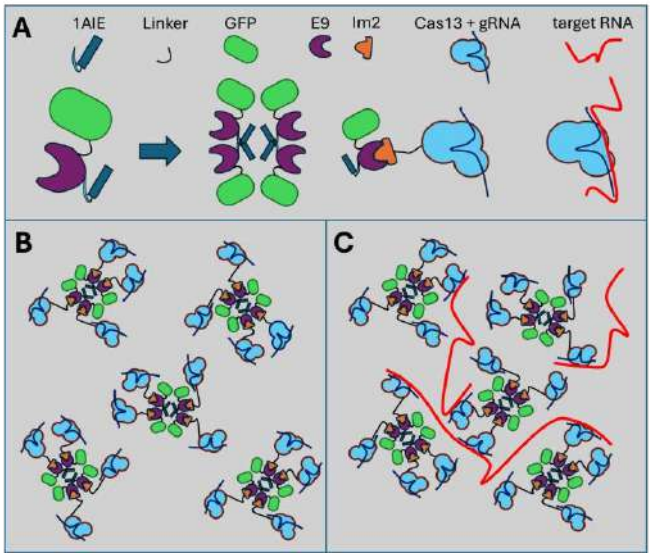

Figure 1. ***Visualisation of components and mechanisms of TRAPS system*** *A. The constructs will form a tetramer, which will function as scaffold. The Cas13 and gRNA are connected through the E9-Im2 toxin-immunity binding to the scaffold structure. The gRNA/Cas13 complex will bind the RNA of interest. B. After expression the TRAPS system diffuses freely through the cytosol.C. In presence of the target RNA a network will form, leading to LLPS.*

V. MATERIALS AND METHODS

ymCherry integration

To introduce *ymCherry* into W303 *S. cerevisiae* gateway cloning was performed. The *ymCherry* cassette was cloned into the pAG304GAL-ccdb plasmid from the pEntry-*ymCherry* plasmid using the LR-clonase reaction. The resulting pAG304GAL-*ymCherry* plasmid was linearized with MfeI and transformed into W303 *S. cerevisiae* for genomic integration into the tryptophan locus.

TRAPS-Cas13 integration

For the functional TRAPS-Cas13 system two fusion proteins and one gRNA cassette, coding for five gRNAs, were introduced into the *ymCherry* containing *S. cerevisiae*. Two individual plasmids were used for the transformation. Both the GFP-E9-Tetramer protein and Im2-Cas13 protein were ligated, introduced into one centromeric plasmid and transformed. pAG416 was used as the plasmid backbone. The cloning was performed by conventional cloning using the SacI and MluI restriction sites in the plasmid. The gRNA coding cassette was similarly transformed using the pAG305 plasmids backbone. The plasmid was linearized using EcoRV and genomically integrated into the leucine locus. The yeast transformation was done with LiAc/Peg chemical transformation method.

Imaging

To specifically activate the RNA production the Gal-Promotor was induced by exchanging the carbon source from glucose to raffinose and finally galactose. The cells were imaged after activation. For imaging a Nikon ECLIPSE Ti2 was used with 1000x magnification for live yeast cell imaging.

Image analysis

Cell fluorescence intensities were obtained by segmenting the cells using Cellpose 2.0 (Stringer et al., 2021) with the cyto3 model and extracting the intensities from the corresponding fluorescence channels. For analysis the average fluorescence intensity of the non-cell areas was subtracted as background.

To test the functionality of the TRAPS system yeast optimized mCherry was integrated into a W303 *S. cerevisiae* under the control of a galactose-dependent promoter, resulting in significant mCherry fluorescence upon galactose induction (Figure 2.). Some fluorescence was observed in the vacuoles of the uninduced cultures, but not in the cytosol (Figure 2.B). In the induced *S. cerevisiae* cells, bright mCherry fluorescence is seen in the cytosol (Figure 2.D).

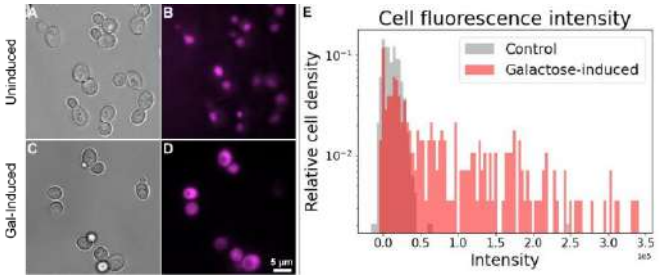

Figure 2. ***Fluorescence microscopy of the mCherry expression.*** ***A-D*** *Brightfield and Fluorescence microscopy images and mCherry channel of the uninduced and induced cultures.* ***E*** *Histogram of summed mCherry fluorescence per cell On the y-axis is the relative density of cells in a logarithmic scale. On the x-axis the sum intensity.*

We introduced the TRAPS-Cas13 fusion proteins to investigate if the mCherry RNA can be captured and contribute to the formation of a condensate. In fluorescence microscopy droplets were observed (Figure 3.). These structures were independent of target RNA or gRNA presence (Figure 3. F,I,L,O).

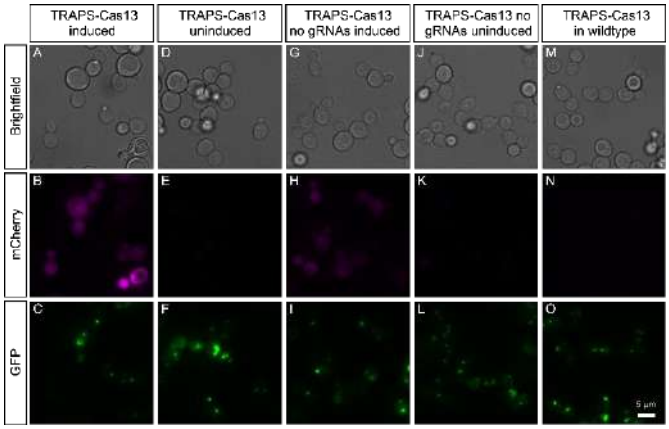

Figure 3. ***Fluorescence microscopy of the mCherry and GFP expression.*** ***A-O*** *Brightfield and fluorescence microscopy images, mCherry and GFP channel of the mCherry RNA induced and uninduced cultures. The gRNAs were targeting the mCherry RNA.*

VI. DISCUSSION

mCherry expression

The strong cytosolic fluorescence signal in the galactose induced samples indicates a successful *ymCherry* integration. If the yeast is continuously grown in glucose containing media no cytosolic mCherry is observable confirming the inactivity of the galactose dependent promoter. Interestingly, a fluorescent signal is observed in the vacuole of the respective cells.

This fluorescence can be attributed to two possible causes. First being, that adenine deficient *S. cerevisiae* strains, like the used W303, generally have a tendency for autofluorescence in the vacuole due to the accumulation of toxic metabolic intermediates in the vacuole (Park et al., 2014). A second possible cause is the accumulation of mCherry degradation products. The respective yeast cultures were grown overnight, diluted in the morning in new media and imaged in the evening. In the overnight culture a scarcity of glucose in the media during the stagnant growth phase can cause activation of the galactose promoter, which activates mCherry production. This overnight expressed mCherry will be degraded during the next hours in new glucose media, but degradation products might still be visible in the vacuole. This is usually unlikely but may happen when the glucose is fully depleted (Harrison et al., 2021).

In conclusion the conditional mCherry expression was achieved, but a promoter leak in the overnight growth is not ideal for the following tests, since this might already activate irreversible condensation of our proteins distorting results. To overcome this, it is necessary to add new media needs every 3-4 hours, even during overnight growth.

TRAPS-Cas13 functionality

The genes coding for the two fusion proteins composing the TRAPS-Cas13 system were transformed into mCherry containing and mCherry deficient yeast stains. Independent of the presence of our mCherry target RNA the system formed a droplet droplet in the cell (Figure 3.). The droplet might be a liquid-like condensate or a more solid-like aggregate. Since the formation of this droplet is independent of the RNA, protein-protein interactions are most likely the driving force. Here the current status of this project is reached and the following conclusions are assumptions.

Due to the RNA independent droplet formation, the observed droplets are most likely aggregates rather than the desired condensates. This assumption is also further supported by the low fluidity observed in the mobility assay.

In the heat-shock response of *S. cerevisiae*, a multitude of chaperone proteins are being expressed, that unfold and refold misfolded proteins. It has been shown that this response is also able to dissolve aggregates (Duennwald et al., 2012). This mechanism was also tested on the putative aggregates, but since no change was observed this approach was not further followed on.

Which of the two proteins is the cause of this aggregation is currently being investigated. It is unlikely that the tetramerization unit is the contributor of this aggregation since the unit has already been proven not to (Heidenreich et al., 2020). The working hypothesis is the aggregation of the Im2-Cas13 units.

Future outlook on the TRAPS platform

To determine and repair the error in the current iteration of the systems proteins are being transformed individually and the tendency to form aggregates is monitored. Additionally, the results of an upcoming western blot might give more insight into the cause of the aggregation. If the assumption that Cas13 is the main contributor is confirmed a new version of the fusion protein will be integrated switching from the current dCas13x variant to a more stable and yeast optimized rfxCas13d variant. Once the initial concept validation is successful, a multitude of further experiments are planned to investigate the efficiency and to adapt the platform to more than just sensing purposes. One important parameter of the system is the RNA copy number threshold at which no condensation occurs. To investigate this, an RNA titration experiment is planned. Additionally, the system will be tested on a native RNA Hfs1, which is highly upregulated during a heat shock response (Hahn et al., 2004). Further experiments like adding a translation initiation or inhibition factors to the scaffold, to regulate translation are also being evaluated.

VIII. CONCLUSION

We are establishing TRAPS as a novel RNA detection platform based on Cas13 driven phase separation. While we already successfully produce condensate-like structures, their formation seems to be independent of target

RNA presence. The current construct therefore needs improvement. Once refined, TRAPS is a promising platform with many possible adaptations to suit different purposes. As a detection platform it is a highly flexible and modular system, making it easy to adjust for different targets. It enables robust real-time *in vivo* RNA detection at low concentrations.

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