Bulk purification of RNA–Protein Complexes

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The ribonome interconnects the proteome and the transcriptome. Specific biology is situated at this interface, which can be studied in bulk using omics approaches. In this entry, we focus on both RNA- and ribonucleoprotein-(RNP) centric methods. These methods can be used to study the dynamics of the ribonome in response to a stimulus. The purpose of this entry is to provide and discuss an overview of the currently available RNA- and RNP-centric approaches to study RNPs.

Keywords: RNA-binding proteins ; phase separation ; RNA-centric ; Ribonucleoprotein complexes

1. Opening Doors to the RNA-Binding Proteome

1.1. Why Study These Proteins? What New Exciting Insights Can Such Study Reveal?

Ribonucleoprotein complexes (RNPs), composed of both RNA molecule(s) and one or more RNA-binding proteins (RBPs), are known to play key roles in cell homeostasis and cell fate by controlling post-transcriptional gene regulation and RNA processing. This can involve gene expression, RNA storage, RNA stability and RNA transport [1]. Until recently, RBPs were classified as such by the presence of one or more RNA-binding domains (RBDs), such as an RNA-recognition motif (RRM), K homology domain (KH), DEAD-box helicase domain, Pumilio/FBF domain, zinc fingers, etc. [2]. However, several research groups have discovered hundreds of RBPs missing such domains, suggesting the limitations of computational prediction [3][4]. The discovery of RBPs lacking any canonical RBD underscores that the number of RBPs is likely underestimated [5][6]. It has been shown that cells adapt to physiological cues through changes in the RBPome, making the study of the dynamics of the RBPome a starting point for many biological questions [7][8][9]. The study of these RNPs could provide important insights into, for example, the regulation and function of long noncoding RNAs (lncRNAs). The mechanism of how Xist, one of the most extensively studied lncRNAs, regulates the silencing of the X-chromosome was recently elucidated by the study of its interacting RBPs [10][11]. Furthermore, the abnormal functioning or expression of RBPs can be linked to multiple diseases [12], as was demonstrated for the hereditary hyperferritinemia-cataract syndrome, arising from a mutated RBP [13]. Additionally, the identification of viral susceptibility genes by studying RNPs could provide new perspectives, as was shown by several research groups [9][14][15][16][17][18]. Opening doors to the RNA-binding proteome is resulting in exciting new insights into a rapidly evolving scientific field.

1.2. The Study of the Ribonome: Where Are We Now?

Over the last two decades, several techniques have been developed to study the interplay between RNA and their cognate RBPs. These methods use an RNA-centric or protein-centric approach and target a specific RNP or an entire group of RNPs. For such a rapidly evolving field, it can be challenging to maintain an overview of the available methods and more importantly how they can be applied. Additionally, standard ways of analyzing, presenting and visualizing the resulting data have yet to emerge.

It has become clear that RNA is more than solely a blueprint to be translated into functional proteins, as it can also function as a scaffold to coordinate and organize protein networks and vice versa. Earlier, protein-centric approaches, such as Cross-Linking and ImmunoPrecipitation (CLIP), were applied to describe these interactions [19][20]. Although successful, these approaches are focused on a single species of protein interacting with one or a diverse group of RNA molecules. However, the question is often complementary but reversed: which proteome interacts with an RNA species?

Despite the recent availability of multiple RNA-centric methodologies discussed in Section 3, only a few specific RNA molecules have been studied extensively, of which the resulting data did not always yield consistent results. Not only does each method have its own strengths and weaknesses, but there are some challenges, involving yield, cost and specificity, common to all. Overcoming these could result in major advances in the field and a better characterization of multiple RNPs, even the ones difficult to study, such as those associated with low-copy-number RNAs. With the recent publication

of organic phase separation protocols such as Cross-Linked RNA eXtraction (XRNAX) [21], orthogonal organic phase separation (OOPS) [22] and Phenol-Toluol extraction (PTex) [23], we address these challenges and propose future experimental strategies that build on these methods.

As mentioned in Urdaneta et al. (2019) and Beckmann (2017), we have to highlight that the term RBP has a historical role in functional RNA biology. Since cross-linking an RNP is based on the physical proximity of the RNA and the protein rather than on the physiological role of the protein, the term RBP becomes broader or a new term has to be introduced, as suggested by Urdaneta et al. Because of this proximity binding, the question of whether the identified binding proteins are functional binders or just spatially close to a given RNA molecule during the time of cross-linking can arise. Together with the fact that a lot is still unknown about the role and importance of numerous transient RNA–protein interactions, it is advisable to experimentally validate identified targets, no matter how stringent the purification procedure [23][24].

2. Isolating the RNA-Bound Proteome to Study RNP Dynamics

Multiple techniques are available to target the RBPome. These methods can be categorized into three main groups: affinity-based-, solid-phase-based and based on organic phase separation. A summary can be found in Table 1.

Table 1. Overview of techniques to isolate the RNA-bound proteome to study RNP dynamics.

Method	RNA Target	Advantage	Disadvantage	Cell Type	Cell Number
Affinity-Based Separation					
RIC [3][25]	Poly(A) tailed RNA	Isolates only mRNA complexes (if subset is of interest) Widely used protocol	Isolates only mRNA complexes Low signal-to-noise ratios Additionally, co-purification of non-cross-linked (free) RNA Can purify off-target RNA containing poly(A) stretches within its sequence	All poly(A) tailed containing organisms	7500 cm^2 HeLa cells [3] 10^7 cells [26] 10^8 cells [27]
e/cRIC [7] [9]	Poly(A) tailed RNA	Isolates only mRNA complexes (if subset is of interest) Better signal-to-noise ratios than RIC	Isolates only mRNA complexes Additionally, co-purification of free RNA Can purify off-target RNA containing poly(A) stretches within its sequence	All poly(A) tailed containing organisms	1–1.3 × 10^8 cells [7] 3 × 15 cm dishes at 80% confluence [9]
CARIC [28]	Newly transcribed RNA	All RNA types RNP monitoring through time	Use of nucleoside analogs Potential co-purification of naturally biotinylated proteins Additionally, co-purification of free RNA	Limited to cell cultures receptive for nucleoside analogs	4 × 10^7 cells
RICK [29]	Newly transcribed RNA	All RNA types RNP monitoring through time	Use of nucleoside analogs Potential co-purification of naturally biotinylated proteins Additionally, co-purification of free RNA	Limited to cell cultures receptive for nucleoside analogs	Not specified
Solid Phase Separation					
2C [30]	All RNPs	Fast and cost-effective method	Contamination of both free protein and free RNA Dependent on the scale of the silica columns A nucleotide size limitation can occur inherent to silica matrices	All cell types and tissue	Not specified

Method	RNA Target	Advantage	Disadvantage	Cell Type	Cell Number
(PAR)-TRAPP [31]	All RNPs	Cost-effective method Scalable protocol	DNA is co-eluted Additionally, co-purification of free RNA A nucleotide size limitation can occur inherent to silica matrices	All cell types and tissue	750 mL of media containing cells at an OD600 of 0.5
VIR-CLASP [32]	Pre-replicated viral RNPs	Study of early-stage viral infection Theoretically adaptable to every type of in vitro transcribed RNA molecule Cost-effective method	The current field of application is a highly interesting but small niche SPRI beads can have size-selective artefacts	Limited to cell cultures receptive for nucleoside analogs	15 cm^2 of cells
Organic Phase Separation					
XRNAX [21]	All RNPs	All RNA types Little free RNA Cost-effective method Easily scalable Good starting point for more specific techniques	Glycoproteins and RNA–protein adducts cannot be distinguished Technically challenging Crude fraction	All cell types and tissue	1 × 10^8 cells
OOPS [22]	All RNPs	All RNA types Cost-effective method Easily scalable	Technically challenging Cannot be used as a starting point for more specific techniques	All cell types and tissue	28.2 cm^2 of 90% confluence
PTex [23]	All RNP >30 bp	All RNA types Little free RNA Cost-effective method Easily scalable Good starting point for more specific techniques	Glycoproteins and RNA–protein adducts cannot be distinguished Technically challenging 25–30% recovery	All cell types and tissue	2 × 10^6 cells

2.1. Affinity-Based Separation

(Enhanced/Comparative) RNA Interactome Capture ((e/c) RIC)

RNA Interactome Capture (RIC) was the first RNA-centric approach developed to address the limitations of in vitro, bio-informatic and protein-centric approaches to identify the mRNA interactome without prior knowledge of the RNA interacting proteins. Cellular RNPs are UV cross-linked, after which the mRNA-bound proteome is purified using oligo(dT) beads and identified using quantitative mass spectrometry [3][4]. Once a protein is enriched in an irradiated sample compared to the non-cross-linked control sample, it is defined as an RBP. Data obtained using this methodology reveal that the eukaryotic mRNA-binding proteome is substantially larger than anticipated. Although RIC is an important technique to identify the mRNA interactome, it also has several drawbacks. Only proteins interacting with poly(A) tailed RNA are captured, and thereby they represent only a part of the whole RBPome, leaving out rRNA-, tRNA-, ncRNA-, lncRNA-, snoRNA- and snRNA-interacting proteins. Consequently, RBPomes of prokaryotes lacking abundant poly(A) tails cannot be isolated using RIC. In addition, the technical noise and experimental variability of RIC still limit the utility of the technique to study the function and dynamics of the RBPome upon environmental and pharmacological stimuli. To deal with these variation issues, enhanced RIC (eRIC) using Locked Nucleic Acid (LNA)-modified probes, allowing the use of more stringent washing conditions, was designed. This optimized protocol reduces the material requirement, improves signal-to-noise ratios (10 times less rRNA and DNA contamination was observed) and enables studying the dynamics of the mRNA interactome upon different experimental conditions [7]. In addition, Garcia-Moreno et al. (2019) developed comparative RIC (cRIC), by altering the existing RIC protocol with the use of SILAC (stable isotope labelling by amino acids in cell culture), enabling the study of the dynamics of the mRNA interactome upon sindbis viral infection. It was shown that a quarter of the mRNA interactome changes upon sindbis infection, of which a few were proven to play a vital role in viral virulence. This research clearly shows the usefulness of RNP capture methods in studying physiologically important systems and cues [9].

RNA Interactome Using Click Chemistry (RICK) and Click Chemistry-Assisted RNA-Interactome Capture (CARIC)

To complement the shortcomings of RIC to capture RNA molecules not harboring a poly(A) tail, multiple methods were designed. Of these, click chemistry-assisted RNA-interactome capture (CARIC) and RNA interactome using click chemistry (RICK) were among the first [33]. RICK and CARIC share a similar approach, only differing in the addition of the photoactivatable 4-thiouridine (4SU) nucleotide analogs and, consequently, also the wavelength of UV cross-linking by CARIC. Instead of targeting a specific RNA sequence or motif, a nucleoside analog (5-ethynyluridine (EU)) is built into the RNA and chemically linked with biotin after a UV cross-linking step [33]. The biotin-linked RNPs can now be isolated using streptavidin-coated magnetic or agarose beads. Since the nucleoside analog is incorporated in all newly transcribed RNA species, a more complete set of the total RBPome is captured in comparison with RIC, which only gathers the poly(A) tailed fraction of the RBPome. Since nucleoside analogs can only be built into newly transcribed RNA from the moment they are administered to the cell culture, one can selectively study the newly transcribed fraction of RNP interaction upon a certain stimulus over time. This feature distinguishes the click chemistry RNA interactome from all other strategies. However, since click chemistry requires the efficient in vivo labelling of RNA, its application may be limited to suitable cell cultures, thereby narrowing its scope. Besides, it should be noted that the streptavidin-based purification can result in the co-purification of rare naturally biotinylated proteins, affecting the reliability of the output.

2.2. Solid-Phase Separation

Complex Capture or 2C Method

The power of the 2C method lies in its simplicity. The method is based on the well-established principle of silica matrices to strongly and specifically retain nucleic acids [34]. Asencio et al. (2018) observed that the silica–RNA interaction is sufficiently strong to also retain UV cross-linked RNA–protein complexes [30]. Since the silica matrix interacts with the RNA molecule in a sequence-independent manner, the whole RBPome is purified. Although no mass spectrometry identification was performed, the bioanalyzer and silver staining results of the retained, washed and eluted RNP fraction points to an enriched level of good integrity RNA–protein complexes. Despite the silver staining showing that large protein bands (range 250 kDa) are co-eluted, more investigation is necessary to elucidate whether all size and/or sorts of biochemically different RNPs are purified with the same efficiency. The XRNAX protocol (discussed later) circumvents this potential problem by pre-treating the complexes with partial trypsin digestion, degrading parts of the protein [21]. The resulting smaller RNPs are probably retained more efficiently and in a more quantitative way, which is essential for the study of RBPome dynamics. Asencio et al. (2018) envisage the use of 2C beyond the validation of RBPs but also to simplify multiple downstream applications to study RNA–protein interactions. By introducing multiple additional purification steps, the RNA-binding domain map (RBDmap), RBPome, RNA binding protein footprint (RBP footprint), RNA interactome and a targeted RBDmap of a single protein could be studied further, as discussed in Section 4.

Total RNA-Associated Protein Purification (TRAPP) and iTRAPP

Similar to the 2C method, TRAPP also makes use of a silica solid phase to retain the RBPome in a non-sequence-specific manner [31]. Instead of silica columns, silicon dioxide particles of 0.5–10 µm are used. The identification of the RBPome of *Escherichia coli* and *S. cerevisiae* was successful and, in addition, Shchepachev et al. could map the dynamics of the RBPome of *S. cerevisiae* upon weak acidic stress using this technique. Silica matrices are known to also retain DNA, possibly giving rise to DNA-interacting contaminants. The combination of the low DNA-protein linking capacity of UV light, denaturing conditions and an extra washing step with a low salt buffer containing 80% ethanol reduces the recovery of contaminating proteins bound to DNA, as clearly shown by the authors. For *S. cerevisiae* samples, different UV types and doses (400/800/1360 mJ/cm^2 at 254 nm and 7200 mJ/cm^2 at 360 nm) were compared for both RNP recovery and for the pathways in which identified proteins function (GO analysis). The quantitative mass spectrometry results clearly show a UV dose-dependent enrichment of RNPs (573/694/1434 proteins) using 245 nm with an overlapping core of 482 proteins in the three UV conditions (400/800/1360 mJ/cm^2). The authors also investigated the RBPome composition using 254 nm (TRAPP) and 365 nm (PAR-TRAPP) UV cross-linking light. Interestingly, GO analysis shows a clear difference for proteins lacking known functions in RNA metabolism/binding, which were more abundant in TRAPP compared to PAR-TRAPP, highlighting the complementary linking character of both UV approaches, as discussed in Section 2.2. Additionally, they could also pinpoint the precise interaction site of 524 RNA-peptide cross-links belonging to 178 proteins using an adapted version of the Xi search engine software [35], coining this approach, iTRAPP.

References

1. Mansfield, K.D.; Keene, J.D. The ribonome: A dominant force in co-ordinating gene expression. Biol. Cell 2009, 101, 169–181.
2. Lunde, B.M.; Moore, C.; Varani, G. RNA-binding proteins: Modular design for efficient function. Nat. Rev. Mol. Cell Biol. 2007, 8, 479–490.

3. Castello, A.; Fischer, B.; Eichelbaum, K.; Horos, R.; Beckmann, B.M.; Strein, C.; Davey, N.E.; Humphreys, D.T.; Preiss, T.; Steinmetz, L.M.; et al. Insights into RNA Biology from an Atlas of Mammalian mRNA-Binding Proteins. Cell 2012, 149, 1393–1406.
4. Baltz, A.G.; Munschauer, M.; Schwanhäusser, B.; Vasile, A.; Murakawa, Y.; Schueler, M.; Youngs, N.; Penfold-Brown, D.; Drew, K.; Milek, M.; et al. The mRNA-Bound Proteome and Its Global Occupancy Profile on Protein-Coding Transcripts. Mol. Cell 2012, 46, 674–690.
5. Gerstberger, S.; Hafner, M.; Tuschl, T. A census of human RNA-binding proteins. Nat. Rev. Genet. 2014, 15, 829–845.
6. Hentze, M.W.; Castello, A.; Schwarzl, T.; Preiss, T. A brave new world of RNA-binding proteins. Nat. Rev. Mol. Cell Biol. 2018, 19, 327–341.
7. Perez-Perri, J.I.; Rogell, B.; Schwarzl, T.; Stein, F.; Zhou, Y.; Rettel, M.; Brosig, A.; Hentze, M.W. Discovery of RNA-binding proteins and characterization of their dynamic responses by enhanced RNA interactome capture. Nat. Commun. 2018, 9, 4408.
8. Sysoev, V.O.; Fischer, B.; Frese, C.K.; Gupta, I.; Krijgsveld, J.; Hentze, M.W.; Castello, A.; Ephrussi, A. Global changes of the RNA-bound proteome during the maternal-to-zygotic transition in Drosophila. Nat. Commun. 2016, 7, 12128.
9. Garcia-Moreno, M.; Noerenberg, M.; Ni, S.; Järvelin, A.I.; González-Almela, E.; Lenz, C.E.; Bach-Pages, M.; Cox, V.; Avolio, R.; Davis, T.; et al. System-wide Profiling of RNA-Binding Proteins Uncovers Key Regulators of Virus Infection. Mol. Cell 2019, 74, 196–211.e11.
10. Chu, C.; Zhang, Q.C.; da Rocha, S.T.; Flynn, R.A.; Bharadwaj, M.; Calabrese, J.M.; Magnuson, T.; Heard, E.; Chang, H.Y. Systematic discovery of Xist RNA binding proteins. Cell 2015, 161, 404–416.
11. McHugh, C.A.; Chen, C.K.; Chow, A.; Surka, C.F.; Tran, C.; McDonel, P.; Pandya-Jones, A.; Blanco, M.; Burghard, C.; Moradian, A.; et al. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. Nature 2015, 521, 232–236.
12. Lukong, K.E.; Chang, K.W.; Khandjian, E.W.; Richard, S. RNA-binding proteins in human genetic disease. Trends Genet. 2008, 24, 416–425.
13. Allerson, C.R.; Cazzola, M.; Rouault, T.A. Clinical severity and thermodynamic effects of iron-responsive element mutations in hereditary hyperferritinemia-cataract syndrome. J. Biol. Chem. 1999, 274, 26439–26447.
14. Knoener, R.A.; Becker, J.T.; Scalf, M.; Sherer, N.M.; Smith, L.M. Elucidating the in vivo interactome of HIV-1 RNA by hybridization capture and mass spectrometry. Sci. Rep. 2017, 7, 1–16.
15. Phillips, S.L.; Garcia-Blanco, M.A.; Bradrick, S.S. Antisense-mediated affinity purification of dengue virus ribonucleoprotein complexes from infected cells. Methods 2015, 91, 13–19.
16. Ooi, Y.S.; Majzoub, K.; Flynn, R.A.; Mata, M.A.; Diep, J.; Li, J.K.; van Buuren, N.; Rumachik, N.; Johnson, A.G.; Puschnik, A.S.; et al. An RNA-centric dissection of host complexes controlling flavivirus infection. Nat. Microbiol. 2019, 4, 2369–2382.
17. Li, Z.; Nagy, P.D. Diverse roles of host RNA-binding proteins in RNA virus replication. RNA Biol. 2011, 8, 305–315.
18. Huh, S.U.; Paek, K.-H. Plant RNA binding proteins for control of RNA virus infection. Front. Physiol. 2013, 4, 1–5.
19. Ule, J.; Jensen, K.B.; Ruggiu, M.; Mele, A.; Ule, A.; Darnell, R.B. CLIP Identifies Nova-Regulated RNA Networks in the Brain. Science 2003, 302, 1212–1215.
20. Ule, J.; Jensen, K.; Mele, A.; Darnell, R.B. CLIP: A method for identifying protein–RNA interaction sites in living cells. Methods 2005, 37, 376–386.
21. Trendel, J.; Schwarzl, T.; Horos, R.; Prakash, A.; Bateman, A.; Hentze, M.W.; Krijgsveld, J. The Human RNA-Binding Proteome and Its Dynamics during Translational Arrest. Cell 2019, 176, 391–403.e19.
22. Queiroz, R.M.L.; Smith, T.; Villanueva, E.; Marti-Solano, M.; Monti, M.; Pizzinga, M.; Mirea, D.M.; Ramakrishna, M.; Harvey, R.F.; Dezi, V.; et al. Comprehensive identification of RNA-protein interactions in any organism using orthogonal organic phase separation (OOPS). Nat. Biotechnol. 2019, 37, 169–178.
23. Urdaneta, E.C.; Beckmann, B.M. Fast and unbiased purification of RNA-protein complexes after UV cross-linking. Methods 2020, 178, 72–82.
24. Beckmann, B.M. RNA interactome capture in yeast. Methods 2017, 118–119, 82–92.
25. Urdaneta, E.C.; Vieira-Vieira, C.H.; Hick, T.; Wessels, H.H.; Figini, D.; Moschall, R.; Medenbach, J.; Ohler, U.; Granneman, S.; Selbach, M.; et al. Purification of cross-linked RNA-protein complexes by phenol-toluol extraction. Nat. Commun. 2019, 10, 990.

26. Zhang, Z.; Boonen, K.; Ferrari, P.; Schoofs, L.; Janssens, E.; Noort, V.; Rolland, F.; Geuten, K. UV crosslinked mRNA-binding proteins captured from leaf mesophyll protoplasts. Plant Methods 2016, 12, 1–12.
27. Castello, A.; Horos, R.; Strein, C.; Fischer, B.; Eichelbaum, K.; Steinmetz, L.M.; Krijgsveld, J.; Hentze, M.W. Comprehensive identification of RNA-binding proteins by RNA interactome capture. Methods Mol. Biol. 2016, 1358, 131–139.
28. Huang, R.; Han, M.; Meng, L.; Chen, X. Capture and Identification of RNA-binding Proteins by Using Click Chemistry-assisted RNA-interactome Capture (CARIC) Strategy. J. Vis. Exp. 2018, doi:10.3791/58580.
29. Bao, X.; Guo, X.; Yin, M.; Tariq, M.; Lai, Y.; Kanwal, S.; Zhou, J.; Li, N.; Lv, Y.; Pulido-Quetglas, C.; et al. Capturing the interactome of newly transcribed RNA. Nat. Methods 2018, 15, 213–220.
30. Asencio, C.; Chatterjee, A.; Hentze, M.W. Silica-based solid-phase extraction of cross-linked nucleic acid–bound proteins. Life Sci. Alliance 2018, 1, e201800088.
31. Shchepachev, V.; Bresson, S.; Spanos, C.; Petfalski, E.; Fischer, L.; Rappsilber, J.; Tollervey, D. Defining the RNA interactome by total RNA-associated protein purification. Mol. Syst. Biol. 2019, 15, e8689.
32. Kim, B.; Arcos, S.; Rothamel, K.; Jian, J.; Rose, K.L.; McDonald, W.H.; Bian, Y.; Reasoner, S.; Barrows, N.J.; Bradrick, S.; et al. Discovery of Widespread Host Protein Interactions with the Pre-replicated Genome of CHIKV Using VIR-CLASP. Mol. Cell 2020, 78, 624–640.e7.
33. Rostovtsev, V.V.; Green, L.G.; Fokin, V.V.; Sharpless, K.B. A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew. Chem. Int. Ed. Engl. 2002, 41, 2596–2599.
34. Boom, R.; Sol, C.J.; Salimans, M.M.; Jansen, C.L.; Wertheim-van Dillen, P.M.; van der Noordaa, J. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 1990, 28, 495–503.
35. Mendes, M.L.; Fischer, L.; Chen, Z.A.; Barbon, M.; O'Reilly, F.J.; Giese, S.H.; Bohlke-Schneider, M.; Belsom, A.; Dau, T.; Combe, C.W.; et al. An integrated workflow for crosslinking mass spectrometry. Mol. Syst. Biol. 2019, 15, e8994.