Advantages and Limitations of UV Cross-linking Analysis of Protein-RNA Interactomes in Microbes

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Abstract

RNA-binding proteins (RBPs) govern the lifespan of nearly all transcripts and play key roles in adaptive responses in microbes. A robust approach to examine protein-RNA interactions involves irradiating cells with UV light to form covalent adducts between RBPs and their cognate RNAs. Combined with RNA or protein purification, these procedures can provide global RBP censuses or transcriptomic maps for all target sequences of a single protein in living cells. Recent development of novel methods has quickly populated the RBP landscape in microorganisms. Here, we provide an overview of prominent UV cross-linking techniques which have been applied to investigate RNA interactomes in microbes. By assessing their advantages and caveats, this technical evaluation intends to guide the selection of appropriate methods and experimental design as well as to encourage the use of complementary UV-dependent techniques to inspect RNA-binding activity.

Introduction

From their synthesis to their degradation, RNAs are escorted by proteins that dictate their fate. In addition to transcription, interactions between RNA and proteins underlie post-transcriptional processes, including maturation, splicing, nuclear export, localisation, stability, translatability, and degradation. RBPs fine-tune gene expression profiles which are essential to maintain cellular homeostasis. Accordingly, disturbance of physiological RNA-protein interactions decreases microbial capacity to rapidly reprogram the transcriptome and adapt to environmental changes [1,2].

Methodological developments during the last decade have vastly increased the technical repertoire to explore RNA-protein interactions *in vivo* . Most methods to detect RNA-protein interactions are based on UV cross-linking, which entails irradiating cells with short wavelength (254 or 365 nm) UV light to induce the formation of covalent bonds between RBPs and directly bound transcripts ("zero distance"; reviewed in [3]). This property makes it possible to isolate RBP-bound RNAs under very stringent and denaturing conditions, greatly reducing noise. Although UV cross-linking is notoriously inefficient and biased towards coupling pyrimidines to a select number of amino acids (reviewed in [3]), it has become a hugely popular tool to study protein-nucleic acid interactions in living systems.

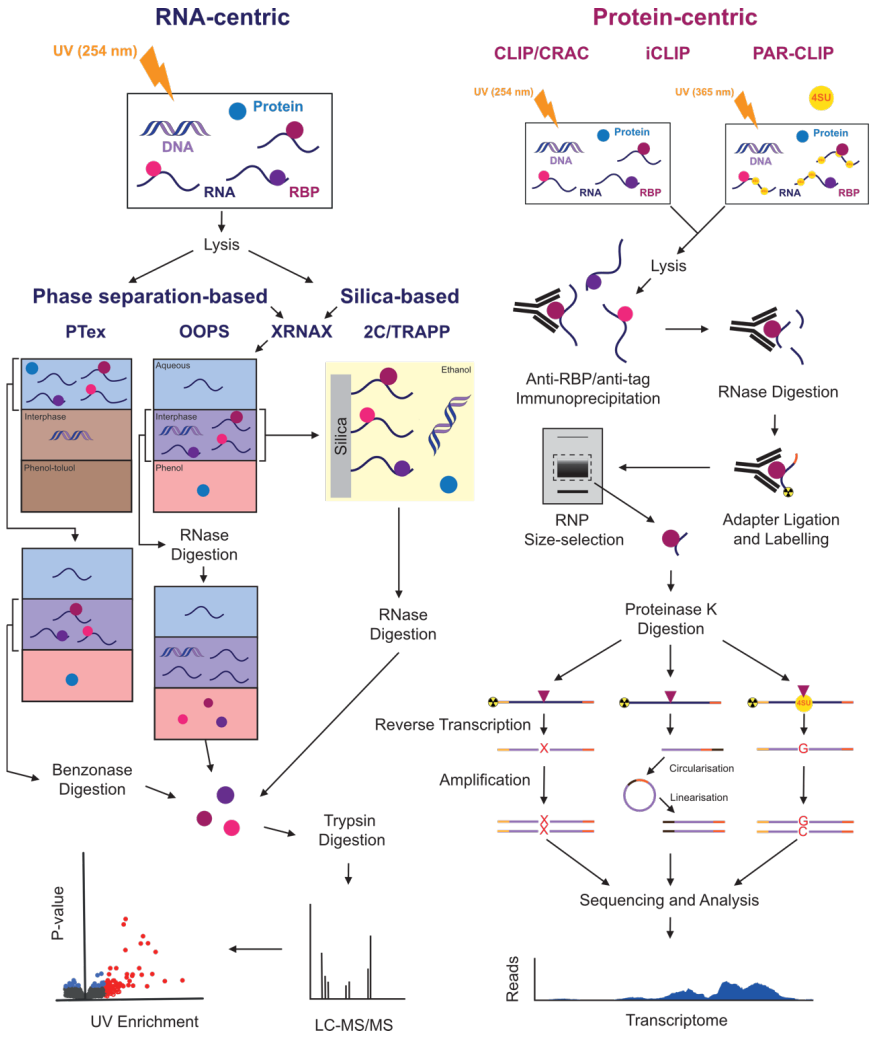
UV irradiation approaches used to study protein-RNA interactions can be classified as RNA-centric, which isolate RNA species to identify cross-linked RBPs, or protein-centric, which capture a specific RBP to study its bound RNA targets (Fig. 1) [4]. Recently, a variety of UV-based high-throughput RNA-centric strategies have characterised the RNA-binding proteome (RBPome) in eukaryotic and prokaryotic microorganisms, which unearthed many novel RBPs. Surprisingly, many of these newly identified proteins lack hitherto known RNA recognition motifs (RRM) or functional links to RNA metabolism. For instance, in multiple studies, metabolic enzymes constituted a prominent fraction of these putative RBPs[5]. However, each of these high-throughput RNA-centric approaches has its own technical caveats and noise levels. Therefore, protein-centric procedures are critical to functionally validate recently discovered RBPs. By combining RNA

and protein-centric approaches one can shed light on how RNA binding affects (i) the life cycle of target transcripts and (ii) the primary function of the associated protein. For example, individual protein-centric analyses have verified that, indeed, some metabolic enzymes moonlight as post-transcriptional regulators or their enzymatic activity is regulated by RNA [6].

This perspective article aims to offer a selective review of these protein and RNA-centric options, discuss their individual strengths and limitations, consider possible technical improvements, and present a complementary workflow for the identification and functional characterisation of novel RBPs in microorganisms.

Defining Microbial RBPomes: Phase-separation vs Silica-based Strategies

The development of several RBPome profiling methods was inspired by well-established whole-cell RNA extraction protocols: the RNA-interactome capture (RIC) procedure employs oligo-dT beads which hybridised to polyadenylated transcripts [7]. Given that technique specifically detects proteins bound to eukaryotic messenger RNAs, it is unsuitable to examine RBPs recognising transcripts lacking (long) poly-A tails (e.g., eukaryotic ribosomal, transfer and small nucleolar RNAs as well as prokaryotic transcripts in general). Click chemistry-based methods (click chemistry-assisted RNA-interactome capture, CARIC; RNA interactome using click chemistry, RICK) have sought to circumvent this drawback by treating cell with synthetic uridine analogues. Once incorporated in newly transcribed RNAs, these analogues can be chemically linked to biotin using click chemistry and immunoprecipitated by streptavidin-conjugated beads[8,9]. More recently, a newly identified click nucleoside analogue was shown to be compatible with metabolic labelling in many bacteria (2'-deoxy-2'-azidoguanosine (AzG)[10]), making it possible to adapt CARIC/RICK based approaches to prokaryotes. Nonetheless, it is important to consider that these nucleotide analogues can be cytotoxic and alter the transcriptome.



Because of the limitations associated with CARIC/RIC, several groups have explored alternative approaches. Grad-seq established the earliest technical foundation for ribonucleoprotein (RNP) identification in bacteria *in vivo* [11,12]. This method, which does not rely on UV, combines density gradient centrifugation with high-throughput RNA sequencing and mass spectrometry to assemble protein and transcript inventories for each fraction. Subsequent grouping of RNA populations with matching sedimentation patterns identifies likely target cohorts that can be further verified by pull-down assays in a few transcripts of each population. Ongoing methodological advances have expanded on this concept to give rise to SEC-seq [13], which pairs high-resolution size-exclusion chromatography with downstream transcriptomic clustering and bait-based validation. Overall, these unbiased approaches are producing hugely valuable data leading, for example, to the identification of a key bacterial RNA chaperone[11]. But, since they do not allow recognition of RBPs in direct association with RNA, UV cross-linking based techniques could be highly complementary. In this respect, the first UV-derived snapshots of bacterial RBPomes arrived with the advent of the orthogonal organic phase separation (OOPS) and phenol-toluol extraction (PTex) protocols, which phase-extract molecules depending on their physicochemical properties (Fig. 1) [14,15]. Concurrently, the protein-cross-linked RNA extraction (XRNAX) approach employed a similar basis in human cells [16].

OOPS applies identical principles to those of standard acid guanidinium thiocyanate-phenol-chloroform RNA extraction procedure. Briefly, upon detergent and phenol addition to the aqueous phase, denatured proteins

and lipids partition to the hydrophobic phenol phase and free RNA molecules migrate to the upper water-rich layer. Due to their intermediate solubility in these conditions, DNA and RNA-protein adducts are predominantly found in the interphase. After trimming the RNA of the RNP complexes in the interphase, downstream phenol extraction rounds enable RBPs recovery from the organic phase. In turn, performing an initial phenol: toluol phase separation at neutral pH, PTex exploits toluol's higher insolubility to separate DNA and lipids, which shift to a phenol-based organic interphase, from soluble RNA, proteins and covalently linked RNPs in the aqueous phase. Following chaotropic treatment of the aqueous phase, phenol addition confines unfolded proteins and unbound RNA to the organic and aqueous phases respectively and, ultimately, allows direct RBPs precipitation from the interphase fraction.

Organic Phase Extraction vs Silica-based Purification

Compared to previous strategies, methods built upon consecutive phase extractions are independent of the sequential features of cognate RNAs and suitable to study the RBPome in prokaryotes such as*Escherichia coli* , *Salmonella enterica* and*Staphylococcus aureus* [2,14,15]. Moreover, in contrast to titration-based techniques, phase partition methods require the lowest number of input cells for putative RBP identification. However, phenol separation can be technically challenging to perform without residual spill over between fractions, which may result in higher background contrasted with some other protocols if not performed carefully. Furthermore, it has been reported that some stable RNPs can be recovered from interphase fractions even without UV irradiation[16]. Organic phase extraction protocols are also inherently incapable of detecting RNA-binding activity in molecules with similar characteristics to those of RNPs; namely, glycoproteins[17]. Nevertheless, a significant advantage of employing organic phase extraction approaches is that they also enrich for cross-linked RNAs, making it possible to globally identify protein-binding sites within RNAs [14,15].

To complement the phase partition protocols, several studies have employed silica-based RNA-centric approaches. Positively charged silica matrices are routinely used to isolate nucleic acids by interacting with the phosphate groups in their backbone. Under stringent conditions, proteins are weakly bound and, consequently, they are easily removed by washes. Importantly, recent work discovered that this charge-based interaction was strong enough to retain RNA-protein adducts in human and yeast lysates [18,19]. As ethanol favours RNA adsorption over DNA binding, the complex capture (2C) and total RNA-associated protein purification (TRAPP) procedures have coupled this isolation protocol to LC-MS/MS-based proteomics (Fig. 1) [18,19]. Subsequently, these techniques have offered simple and quick alternatives for RBPome profiling in human cell lines, *S. cerevisiae* , *E. coli* and *S. aureus*[2,18,19]. It would be interesting to combine GRAD-Seq and SEC-seq with RBPome indexing methods as we envision that the fractionation of the cell lysates would further increase the sensitivity of RBPome approaches.

Dealing with Noise

In contrast to organic phase extraction approaches, silica-based strategies typically yielded higher numbers of significantly enriched RNA-binders. In fact, whereas prior reports had estimated RBPs to conform around a tenth of eukaryotic and bacterial proteomes[7,14], silica-based findings consistently record portions between 20-30% [2,19]. It is plausible that spurious proteins co-purify with RNPs if, like histones or nucleotide-binding enzymes, they bind to DNA or short nucleotides[20]. However, many of these proteins were also abundantly detected in datasets stemming from organic phase separation experiments, which cannot detect RNA-binding in transcripts shorter than 30 nucleotides in length [15]. Regardless, we strongly recommend testing a range of UV irradiation treatments and selecting the lowest possible dose when studying protein-RNA interactions[19]: high levels of UV not only cause substantial protein and RNA degradation [2,21], but also can cross-link proteins to DNA [20]. Thus, treating lysates with DNase is also advisable. Notably, although 2C and TRAPP indeed generate larger pools of statistically enriched proteins, they generally require a larger number of input cells to do so. This constitutes a technical cost for the poorer binding capacity of silica-based capture compared to self-contained phase separation systems.

Despite the obvious methodological differences as well as the advantages and shortcomings which they prompt, silica-dependent and organic phase separation-based produced remarkably similar results for *S. au-*

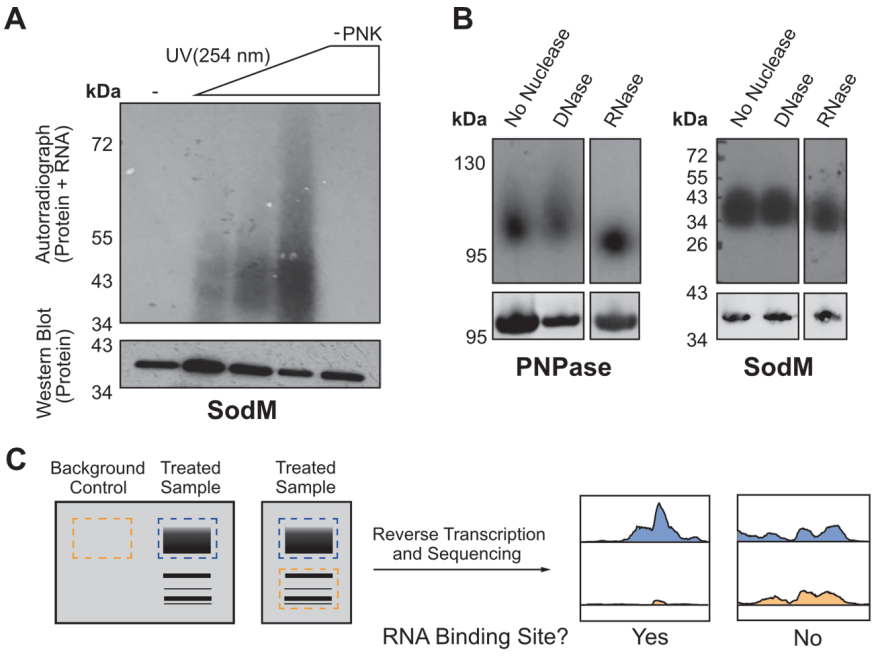
reus [2]. Together with individual validation of the RNA-interaction status of some novel RBPs, this first direct comparison underscored that both approaches are fit for holistic interrogation of microbial RBPomes. In fact, the combination of both strategies could improve the current individual performance of both methods. For instance, in the XRNAX protocol, mammalian RNPs were coarsely partitioned from other cellular components using phenol extraction and further enriched using a silica-conjugated platform, which contributed to reduce noise (Fig. 1) [16].

Protein-centric Approaches: Cross-linking and Immunoprecipitation

Even though detection of RBPs in datasets derived from several RNA-centric RBPome studies is frequently a reliable indicative of RNA recognition, candidate RBPs should not be considered *bona-fide*RNA-binders until they have been shown to bind RNA in their native systems, ideally using a variety of orthogonal *in vitro* and*in vivo* methods. The discovery of non-canonical RNA-binding activity among metabolic enzymes and proteins which are known to bind DNA emphasises the importance of verifying direct RNA binding and excluding false-positives introduced by experimental artifacts. Furthermore, protein-centric studies can provide pivotal evidence for determining the physiological role of such RNA-binding events.

The most widely used techniques for globally identifying the RNAs bound to RBPs are CLIP and related protocols, such as CRAC, eCLIP, iCLIP and PAR-CLIP [22–26]. Like RBPome capture, these approaches rely on UV cross-linking of RBPs to their target transcripts. To enrich for the cross-linked (and, therefore, likely directly bound RNAs), immunoprecipitations are generally performed under (semi-)denaturing conditions [22–25]. Following RNase trimming of unshielded ribonucleotide sequences, adapter ligation and proteinase K digestion of the preserved RNPs, high-throughput sequencing allows transcriptome-wide mapping of the sites to which the tested RBP was binding (Fig. 1). Despite its technical power and widespread use, cross-linking and immunoprecipitation (CLIP) presents some limitations. A family of CLIP methods has emerged to provide solutions for some of these challenges in specific biological contexts [27].

Optimising the UV Irradiation Times Is Critical



A challenge of CLIP procedures is, again, their dependence on UV exposure to generate RNA-protein adducts. As outlined above, *in vivo* UV radiation is particularly inefficient: it usually takes minutes to

complete and only about 1-5% of RNPs of interest are cross-linked[28]. Hence, some CLIP variants often require relatively large number of cells. Systematically poor cross-linking performance could be attributed to the impossibility of creating adducts with some amino or intrinsically limited UV penetration in certain cell types or growth media [2,15]. Recently, we developed a much-improved UV cross-linker (Vari-X-linker; UVO^3 [1,21]) that greatly increases the cross-linking efficiency in actively growing cells during markedly shorter time spans (seconds)[1,21]. However, the fastest and most efficient way to cross-link proteins to RNA is using UV lasers [29], although setting up such a system can be prohibitively expensive. Another major advantage of these rapid cross-linking devices is that they enable the monitoring of very dynamic changes in protein-RNA interactions, such as those occurring during stress responses, at high temporal resolution [1,21,29,30].

To quantify RNA-protein cross-linking efficiencies, most protocols attach radioactive phosphates or fluorescently labelled oligonucleotides to 5' ends of the cross-linked transcripts. The RNP complexes are subsequently resolved by denaturing PAGE (Fig. 2) and the RNA can then be visualised by autoradiography or fluorescent imaging. Recently, we performed such radiolabelling analyses on a variety of RBPs identified in our *S. aureus* RBPome analyses. While the vast majority of the proteins tested detectably cross-linked to RNA, we learned that only for those proteins for which we observed very strong radioactivity signals (1 to 3-hour exposure of phosphoimager screen/film) were we able to obtain high-complexity complementary DNA (cDNA) libraries. It is also important to point out that the radiolabelling assay can generate false positive signals: radioactive labelling of cross-linked RNA involves an incubation step with T4 polynucleotide kinase (T4 PNK). However, it is possible that during this reaction the RBP of interest becomes radiolabelled by autophosphorylation or co-purified host kinases[31]. Consequently, we would always advise including control reactions with non-irradiated cells or leaving out PNK altogether (Fig. 2A).

As well as informing the choice of UV exposure, preliminary signal evaluation provides a strategy by which to filter out proteins with no or little RNA-binding activity. As outlined above, UV can also cross-link proteins to DNA [20]. Accordingly, we strongly recommend performing control experiments where the cross-linked RNPs are incubated with increasing concentration of RNase I/A or DNase I. If the purified RNP indeed contains cross-linked RNA, higher concentrations of RNase should reduce the smearing of the bands corresponding to RNPs (Fig. 2B; PNPase). Alternatively, the intensity of those containing DNA-protein duplexes would only be resolved by DNase digestion. We also found that not all cross-linked proteins, such as the superoxide dismutase SodM, respond to either DNase or RNase treatment (Fig. 2B). This suggests that these proteins generally bind short nucleic acid fragments, which will make the library preparation steps more challenging.

Mapping the UV Cross-linking Sites in RNA

To be able to sequence the cross-linked RNAs, they need to be converted into cDNAs. For this purpose, the cross-linked protein is removed using proteinase K. However, this treatment does not remove amino acids cross-linked to the RNA, which can significantly impact the processivity of the reverse transcriptase (RT) during cDNA synthesis. The cross-linked amino acid residues generally cause the RT enzyme to drop off [32], yielding truncated cDNAs (Fig.1, iCLIP[26]). Nevertheless, depending on the reaction conditions, the reverse transcriptase can introduce mutations in cDNAs at the site of cross-linking (Fig. 1, CRAC [23]), especially when using highly processive RT enzymes and/or manganese-supplemented buffers [33]. UV cross-linking sites on the RNA can subsequently be determined at nucleotide resolution by mapping the nucleotide positions where the RT fell off, or by mining the data for mutations within reads. Alternatively, photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) uses photoactivable 4-thiouracil or 6-thioguanine to enhance the cross-linking efficiency and use T-to-C or G-to-A substitutions as a proxy for RNA cross-linking (Fig. 1)[25]. Conveniently, the PAR-CLIP protocol has been modified for some microorganisms, such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*[34,35], where the technique is now routinely applied. However, adapting such metabolic labelling methods to prokaryotic model systems has proved to be significantly more challenging[10], which may explain why (at the time of writing) only a single manuscript describes the application of PAR-CLIP in bacteria [36]. Additionally, prolonged exposure to the mentioned nucleobase analogues can cause cellular toxicity[10,26].

Dealing with Experimental Noise

The applicability of CLIP-derived procedures is contingent to the extent of their background. A general assumption of these protocols is that random binding is hampered by the harsh washing steps succeeding immunoprecipitation. Moreover, electrophoretic resolution of the RNPs under study ensures an additional enrichment step with respect to adventitiously co-partitioned RBPs. Preferred control samples have normally been acquired from cells where the gene encoding the RBP of interest is knocked out, untagged or which have not been UV treated (Fig 2B-C) [22,23,27,37]. In principle, these samples are not expected to produce any noticeable signal upon SDS-PAGE visualisation. Consequently, libraries emerging from the sequences in the areas corresponding to the expected migration of the RNP under study should contain 100-fold less unique cDNAs than those resulting from fully treated samples [37]. Nonetheless, meta-analysis of 30 published CLIP datasets identified widespread and replicable background reads across most of them [38]. To quantify technical background, prior work sequenced libraries proceeding from RNA-protein adducts that did not migrate with the duplexes of interest (Fig. 2C)[38]. Adjusting output signals to those originated by background characterisation was shown to dramatically improve the identification of RNA recognition sequences [38]. Based on relevant control samples, background correction can indeed statistically disregard binding events resulting from non-specific transcript cross-linking to incidentally proximal RBPs of interest, co-purified RNPs and RNA aptamers adopting an epitope-mimicking conformation.

A number of CLIP-related approaches have been developed that have been specifically designed to further improve signal-to-noise ratios. These methods take full advantage of the fact that cross-lined RNPs can withstand highly denaturing purification conditions. These techniques include cross-linking and analysis of cDNAs (CRAC), ultraviolet crosslinking and affinity purification (uvCLAP) and denaturing CLIP (dCLIP). All of these protocols employ (tandem) affinity-based purification to recognise specific epitope tags that are compatible with denaturing purification conditions and/or strong detergents (e.g., HIS-tag and streptavidin-binding peptides sequences; Fig. 1)[23,39,40]. Admittedly, epitope tagging can alter the stability, expression, and RNA-binding capacity of the RBP of interest. Advantageously, however, specific tag recognition-based partition spares the need for high-quality antibodies, which are not readily available for less well studied model organisms. Accordingly, these approaches can considerably reduce background reads in resulting cDNA libraries. This property also makes them good alternatives to inspect scarcer RNPs, namely yeast pre-ribosomal complexes or those encompassing non-professional RBPs [2,23].

Alternatives to UV irradiation

Although beyond the scope of this article, we feel that it is important highlight recent approaches that have striven to surmount the issues associated with using UV to study protein-RNA interactions. On the one hand, techniques such as TRIBE (targets of RBPs identified by editing) or STAMP (surveying targets by APOBEC-mediated profiling) fuse RBPs of interest to RNA-editing enzymes that introduce nucleotide modifications as a proxy for RNA binding events [41,42]. While these strategies minimise handling and input amounts, tagging proteins with modifying enzymes could jeopardise physiological activity and expression of the RBP or induce cytotoxicity by uncontrolled transcriptomic hyperedition. On the other hand, proteins can also be chemically cross-linked to RNA. Traditionally, formaldehyde is used for this purpose [43], but this treatment also links proteins to other proteins and DNA nucleotides located within 2 Å[4,43]. As a result, several groups have begun to develop more specific chemical cross-linkers that appear to have higher cross-linking efficiencies compared to UV (e.g., NHS-diazirine (SDA) and AMT-NHS [44,45]). Yet, a limitation of these reagents is that they only react with lysine residues and, therefore, these chemicals do not fully circumvent the biases associated with UV irradiation. Nevertheless, this work represents a significant advancement in the field, and we hope that it will spur the development of a wide array of chemical cross-linkers.

Unlike UV, which tends to yield RNA-protein duplexes enclosing single-stranded RNA (ssRNA) species, chemical cross-linkers reportedly display uniform adduct formation patterns for single- and double-stranded RNA targets [45]. We foresee that this feature will nurture future technical advances to investigate RNA-RNA interactions. To date, even despite its ssRNA bias, UV cross-linking has enabled successful retrieval of RNA-RNA associations from immunoprecipitated RBPs harbouring such interactions. Soon after being identified

as a suitable tool to analyse ncRNA-mRNA interactions from yeast RNPs [23], CRAC and other existing CLIP-based protocols were amended to favour intermolecular ligation of complementary RNA species in RNA-RNA base-pairing hubs[46]. Resulting methods, such as CLASH (cross-linking, ligation and sequencing of hybrids), hiCLIP (RNA hybrid and individual-nucleotide resolution CLIP), and RIL-seq (RNA interaction by ligation and sequencing) [46–48], have used RBPs chaperoning RNA-RNA interactions as bait to map and functionally characterise non-coding transcripts. So far, these procedures have been used in systems including mammalian cells, *E. coli* , Salmonella and *S. aureus* [47–55], which unravelled large ncRNA-RNA interactomes in these organisms. Eventually, customising present protocols to replace UV irradiation by chemical cross-linking could verify and expand prevailing knowledge on the microbial and metazoan RNA-RNA interactome.

Conclusion

UV cross-linking lies at the core of several techniques to study RNA interactions. Subsequent formation of covalent bonds ensures maintenance of the complex and facilitates isolation of RNPs. Purifying the transcriptome to capture protein-RNA interactions has spurred the characterisation of the RBPome in an increasing number of microorganisms. Inevitably, the chance of recovering enriched adducts between proteins and hypothetical transcript targets are higher when whole-cell RNA species are used as partitioning baits. Since transcriptome-wide indexing of RBPs is more likely to yield false positives, it is crucial to validate putative RNA-binding activity using protein-centric approaches.

The advantage of producing validatory RNA maps is two-fold. Firstly, interpreting RBP binding profiles can help to unravel their role and its underlying mechanism. Secondly, considering that harsher purification is generally possible in UV requiring methods, these tend to produce less background than their non-UV dependent counterparts. However, cross-linking efficiency and biases may result in false positive signals arising from phosphorylation of DNA or the RBP itself. Furthermore, it is also plausible that the abundance, localisation, or function of a given protein foster consistent but inconsequential RNA cross-linking. As discussed, these situations should become apparent in appropriate downstream functional explorations. Nevertheless, including the mentioned control experiments could economise the efforts invested in characterising proteins which were circumstantially forming duplexes with RNA in previous datasets.

Methodological expansion of these protocols could provide insight into the biology of other components of the RNA interactome. As an illustration, we have referred to CLASH and related protocols, which have specified several RNA-RNA interactions as well as their regulatory functions. Despite not having been reviewed here, an evolving array of techniques has also been using cross-linking and immunoprecipitation to study how the distribution of RNA modifications shape RBP occupancy[56,57]. Ultimately, this interplay of experimental tools will empower a confident understanding of protein-RNA interactions and their implications in wider biological systems.

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