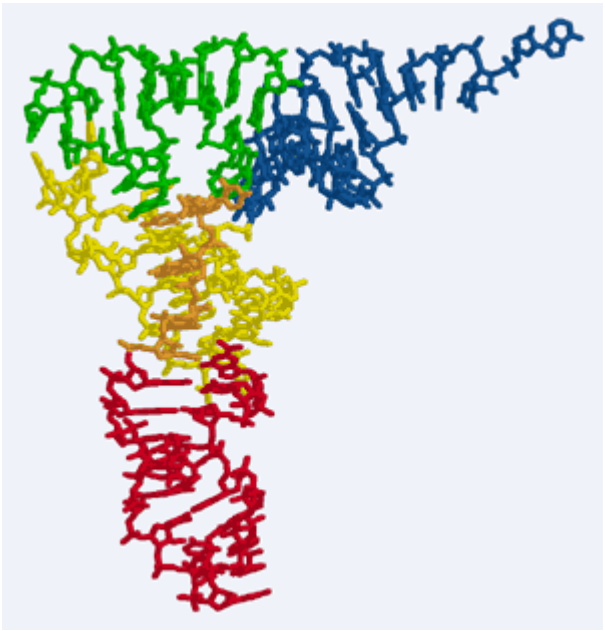
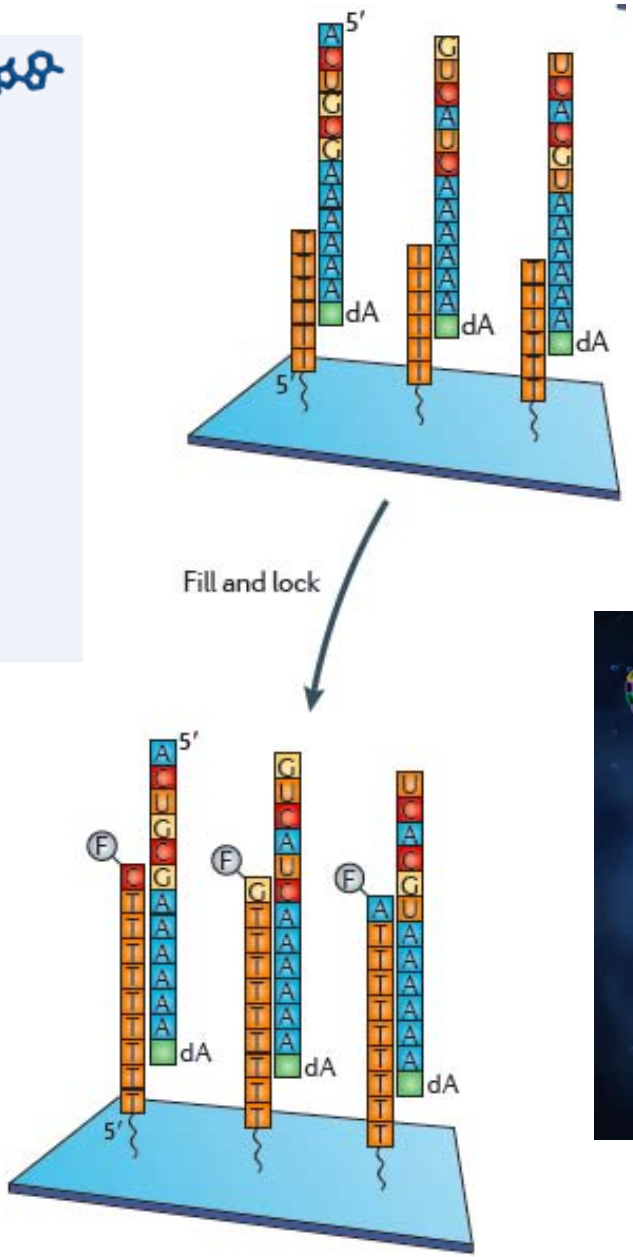
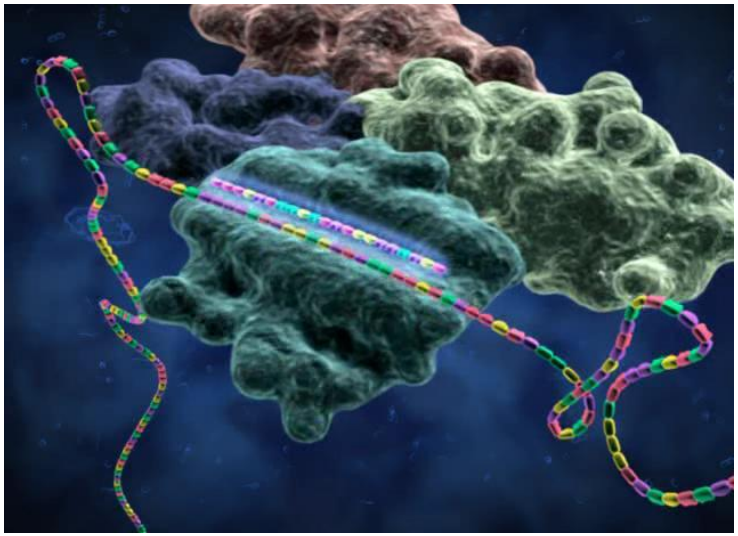
GLOBAL ANALYSES of RNPs







OLD-FASHIONED BIOCHEMICAL PURIFICATION

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 249, No. 18, Issue of September 25, pp. 5963–5970, 1974
Printed in U.S.A.

Isolation, Structure, and General Properties of Yeast Ribonucleic Acid Polymerase A (or I)

(Received for publication, December 28, 1973)

JEAN-MARIE BUHLER, ANDRÉ SENTENAC, AND PIERRE FROMAGEOT

From the Service de Biochimie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91 190 Gif-sur-Yvette, France

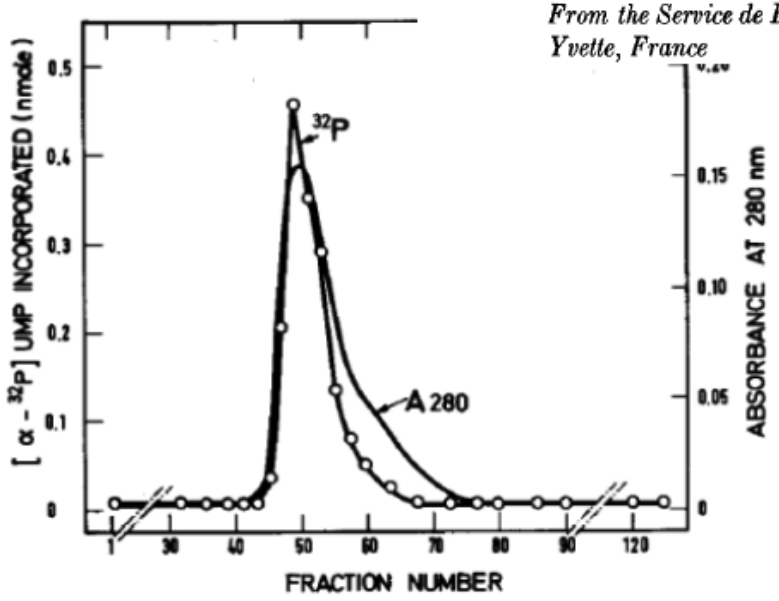


FIG. 1. DEAE-cellulose column chromatography. Fraction 3 (15 ml, $A_{280\ nm}$ 0.8) was applied to a column (5 cm^2 × 16 cm) of DEAE-cellulose and eluted as described in the text. Fractions of 3 ml were collected and assayed for RNA polymerase activity on 10-μl aliquots for 10 min under standard conditions.

TABLE I

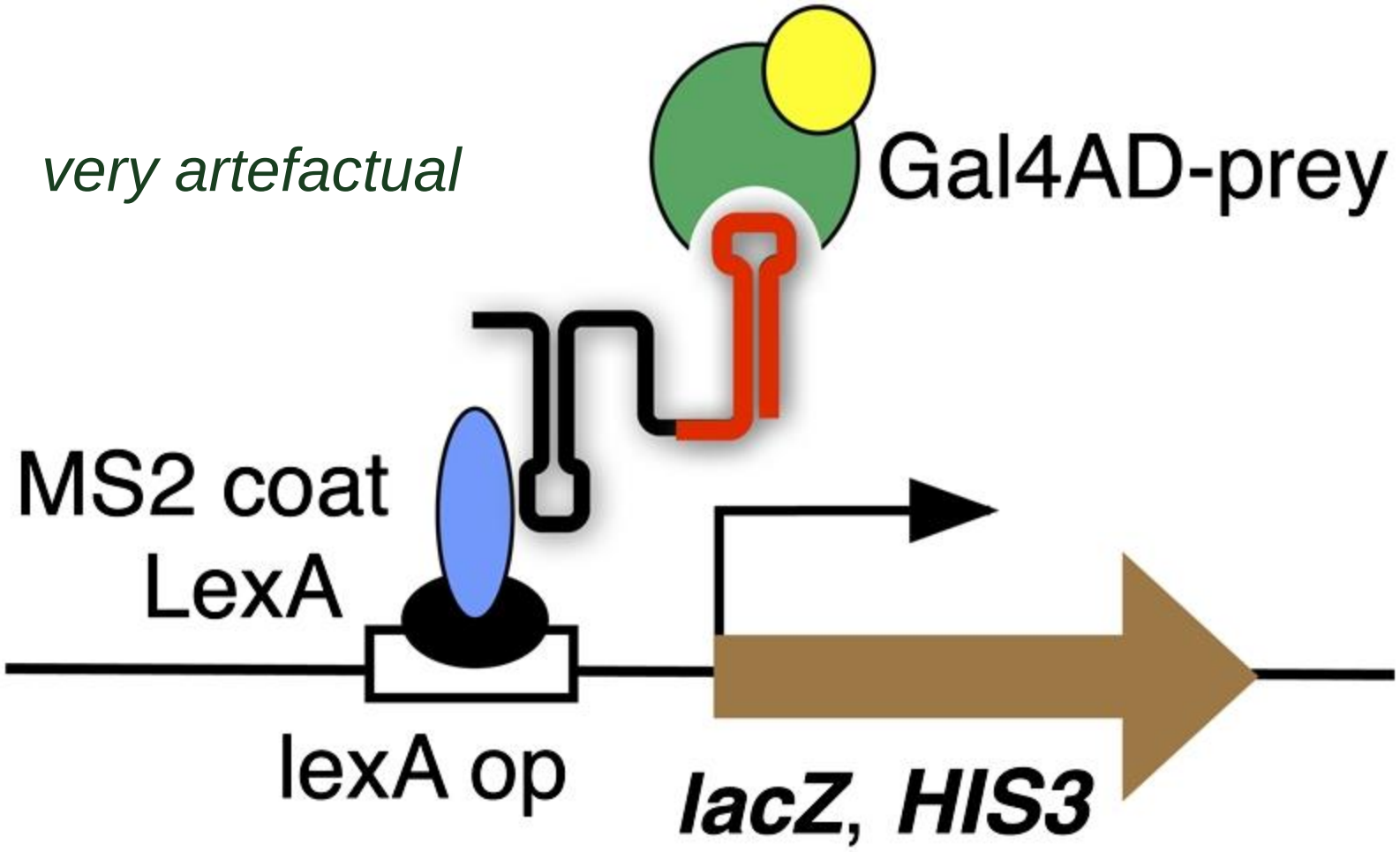
Summary of RNA polymerase A purification

Values are given for 300 g of yeast cells.

Fraction or step in purification	Volume	Proteins	Total activity	Specific activity
	ml	*mg*	*units*	*units/mg*
1. High speed centrifugation..	530	2,300	21,000	0.9[a]
2. Phosphocellulose batch....	290	185	38,000	203
3. DEAE-cellulose batch.....	300	21	25,000	1,200
4. DEAE-cellulose chromatography.....	30	2.5	3,000	1,200
5. Glycerol gradient.....	5	0.5	900	1,800

[a] RNA polymerase A and B are not separated at this stage.

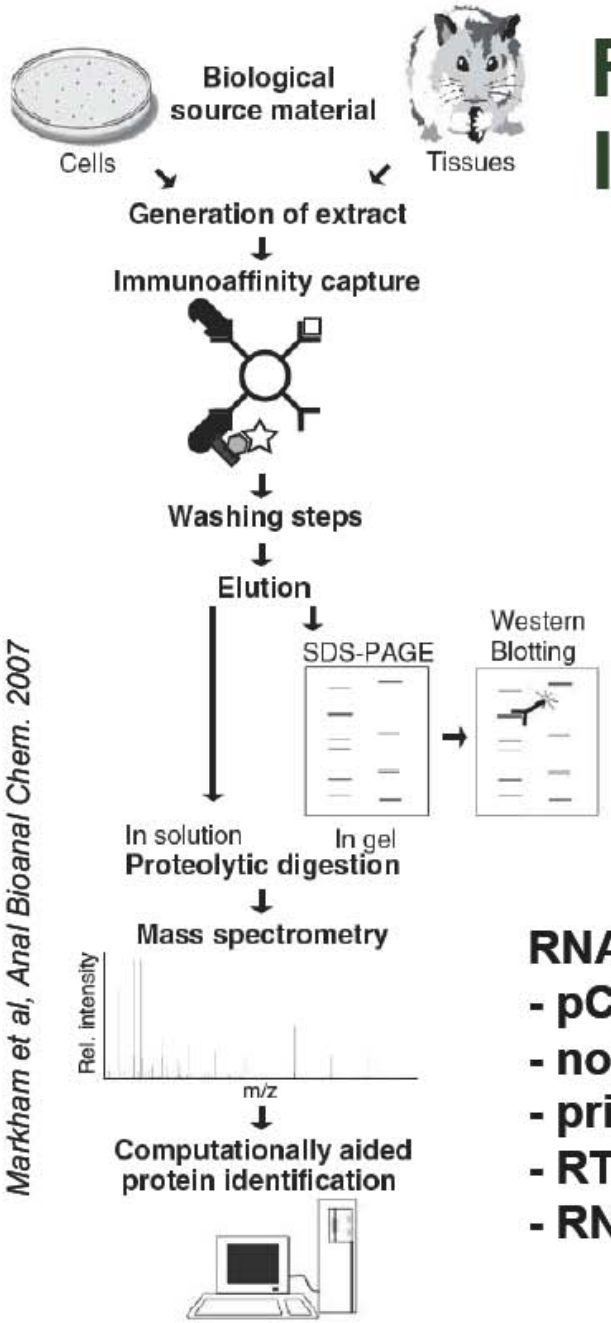
GENETIC SCREEN- YEAST THREE HYBRID



The RNA insert (red) is expressed in the context of RNA vector sequences (black) tethered upstream of *lacZ* (brown) and *HIS3* reporter genes via a MS2 coat–LexA fusion protein (blue and black). Gene activation depends on binding of the Gal4 activation domain (yellow) –prey fusion protein (green).

RNP IMMUNOPRECIPITATION
IP, co-IP

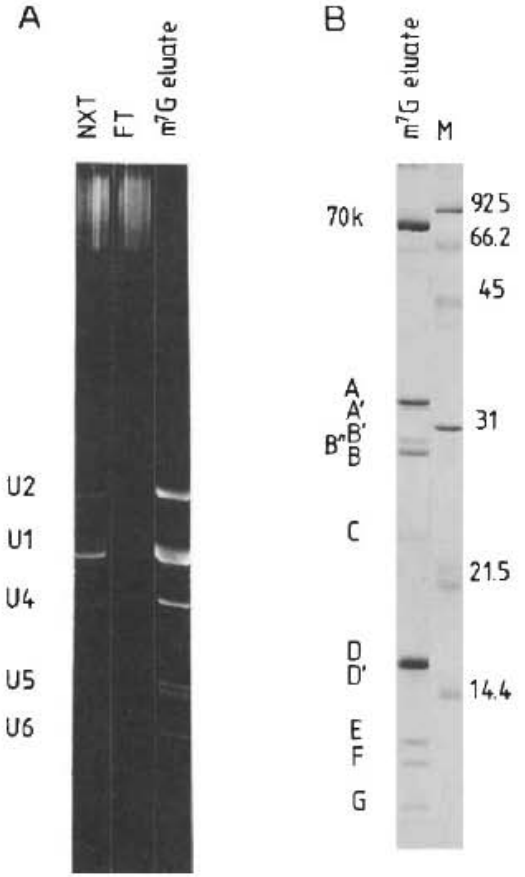
With specific antibodies or using tagged proteins



Markham et al, Anal Bioanal Chem. 2007

RNA analysed by:
- **pCp labeling (3' end)**
- **northern blot**
- **primer extension**
- **RT-PCR**
- **RNASeq**

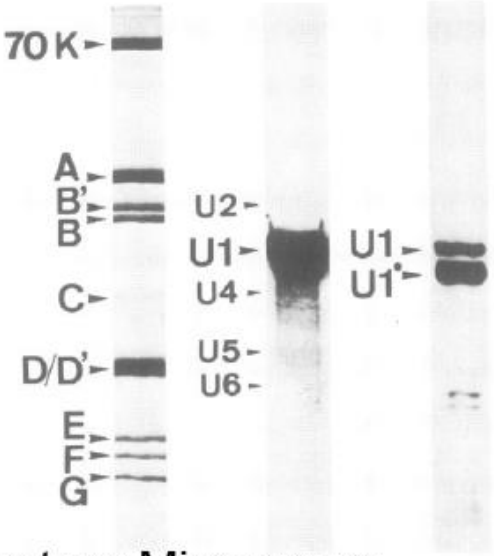
U snRNPs with anti-TMG cap antibody



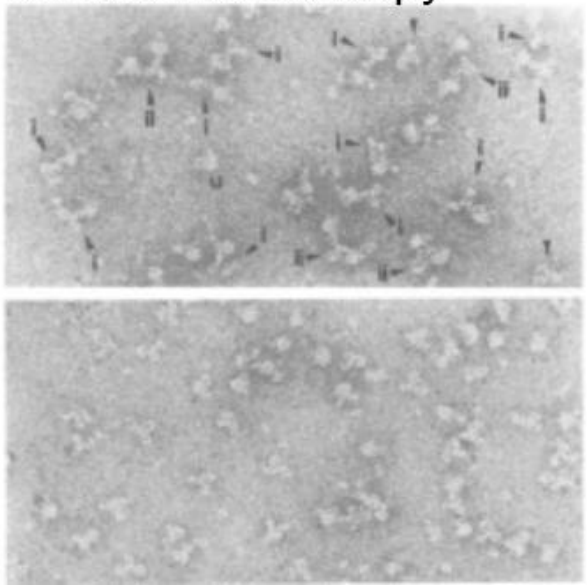
Bochnig et al, Eur. J Biochem. 1987
(Luhrmann's lab)

IP of U1 snRNP with anti-70K Ab (U1 specific protein)

Immunoaffinity +ion exchange



Electron Microscopy



IP of snRNPs with anti-TMG cap Ab

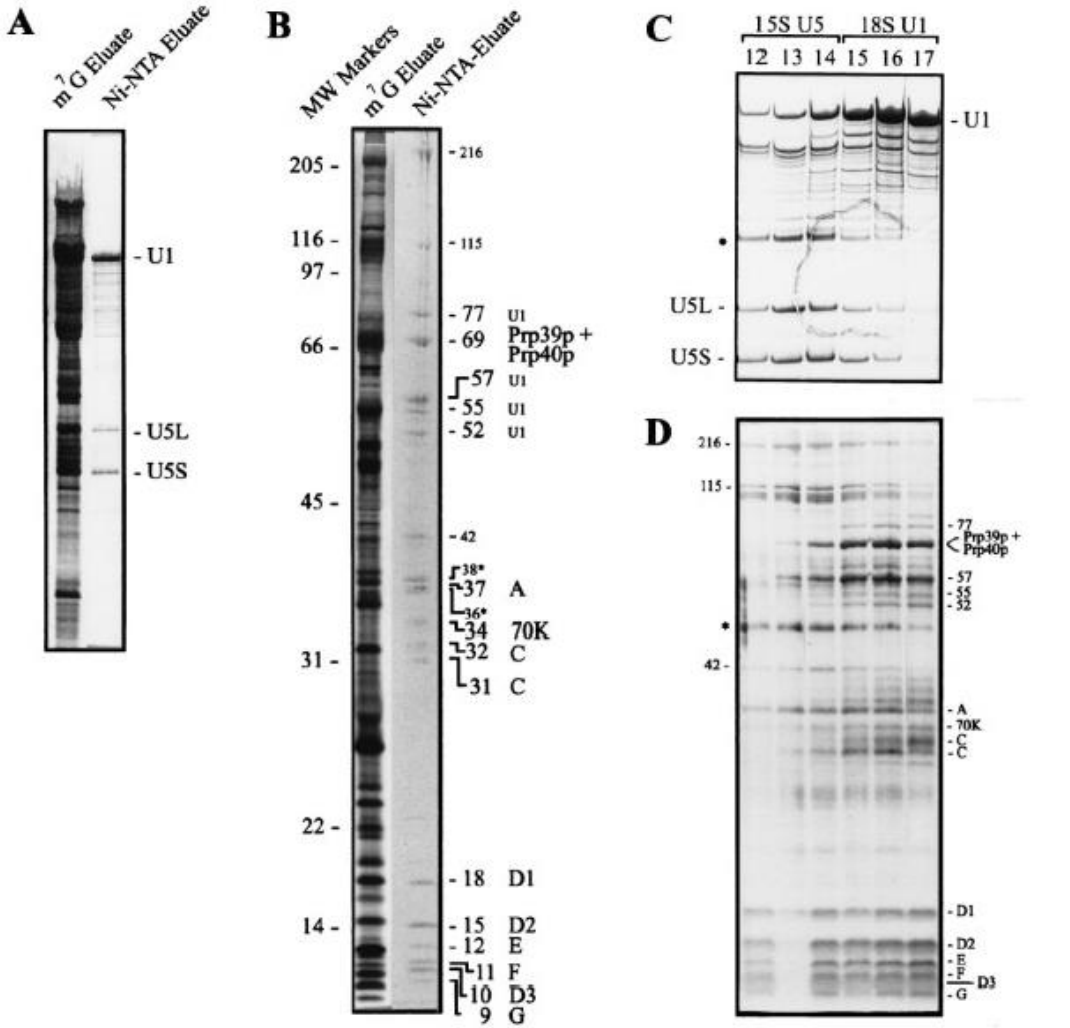
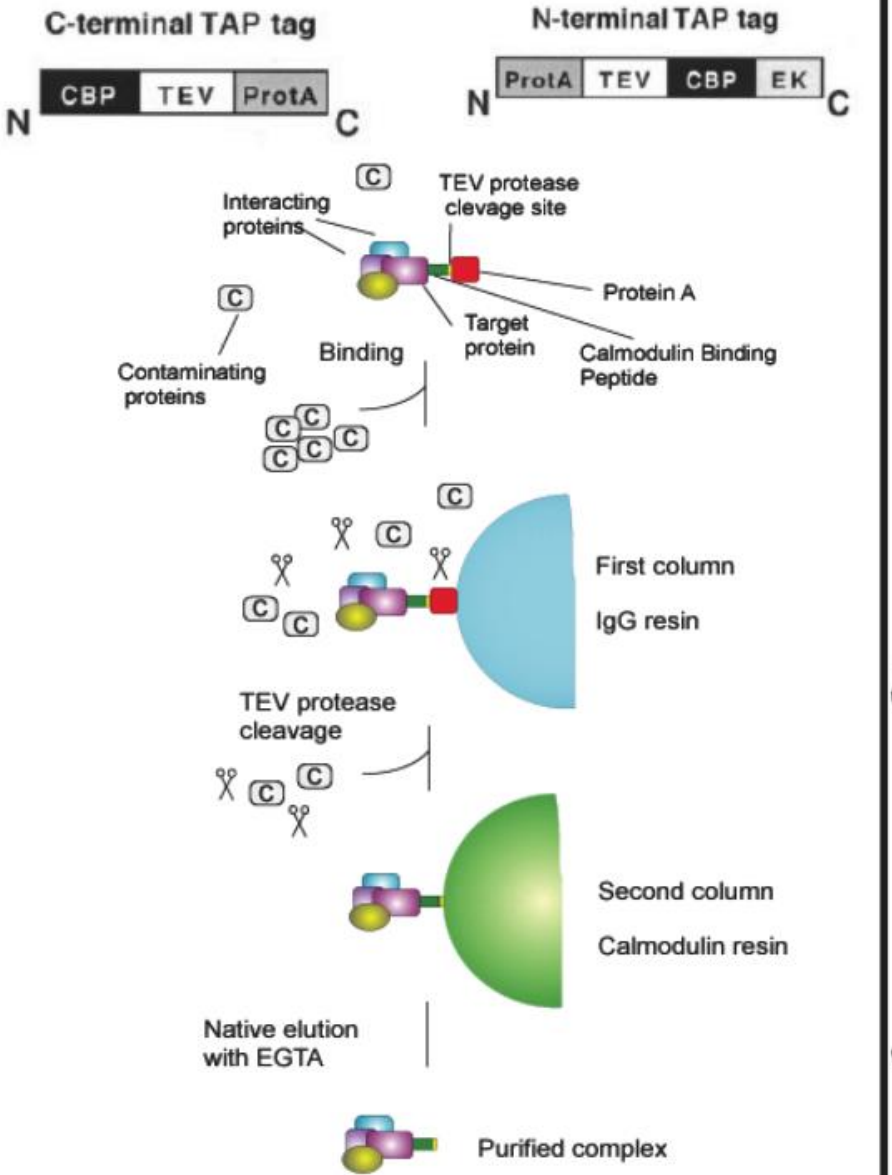
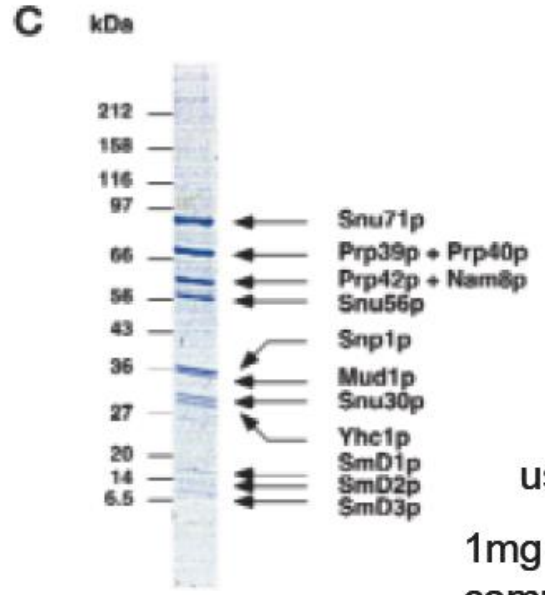
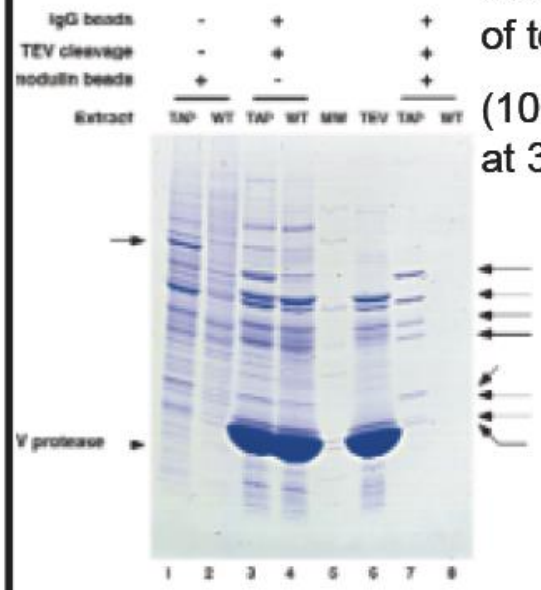


FIG. 1. Purification of U1 snRNPs from *S. cerevisiae*. (*A*) Silver staining of snRNAs eluted from anti-m_3G-cap (m^7G eluate) and Ni-NTA affinity

TANDEM AFFINITY PURIFICATION (TAP)





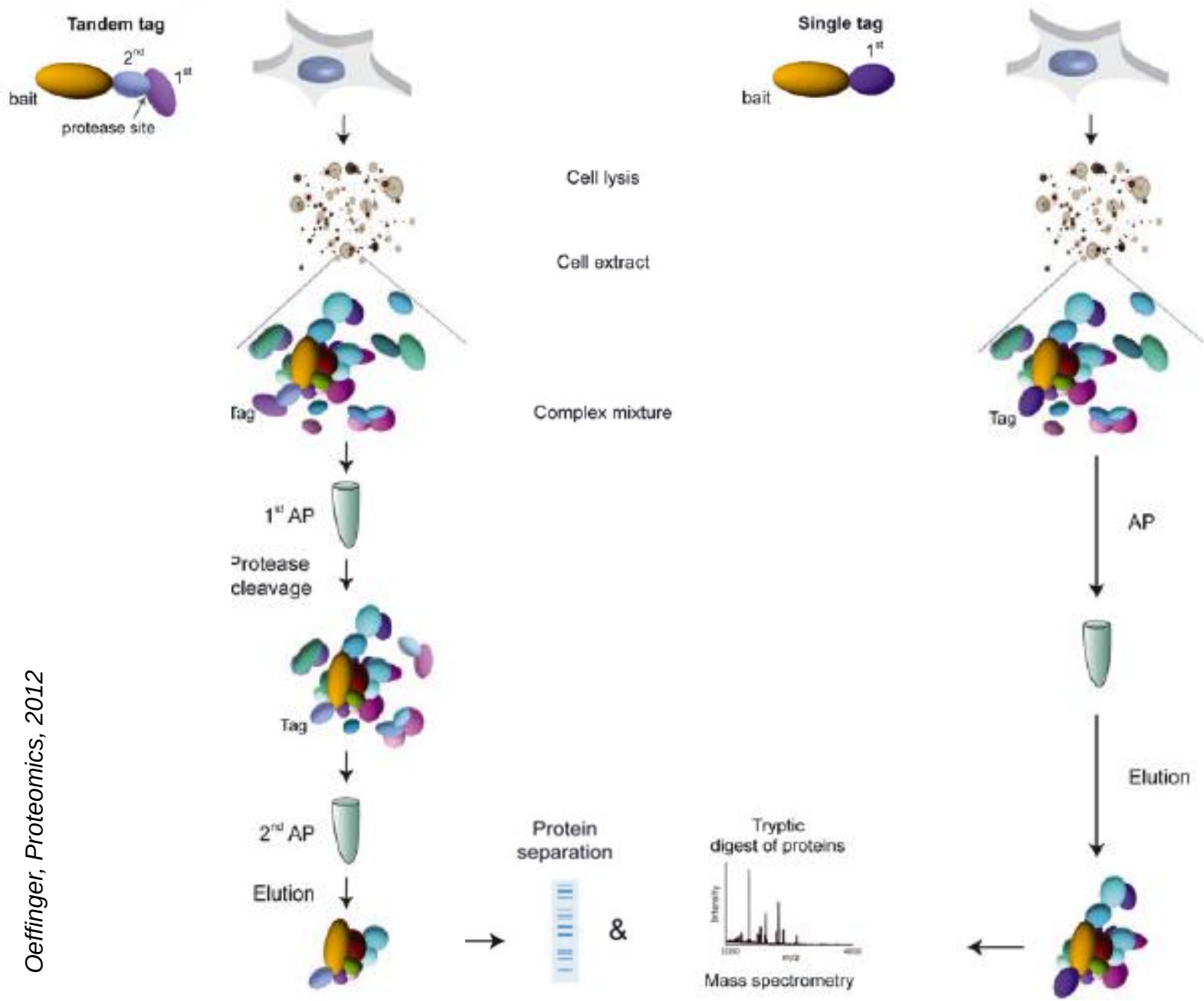


usual yield:

1mg of protein complex from 300mg of total protein

(100 000 X purification at 30% efficiency)

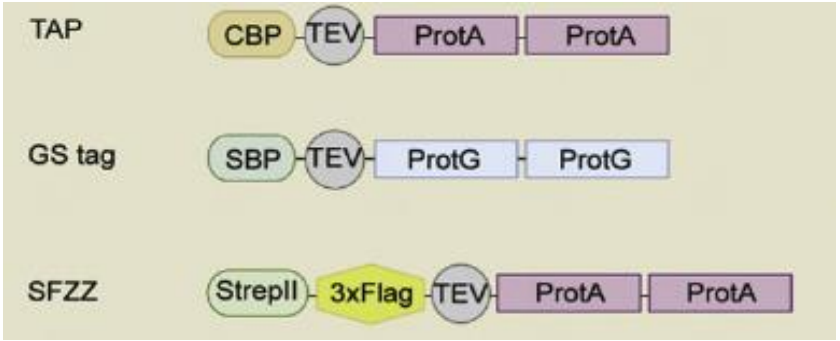
AFFINITY PURIFICATION



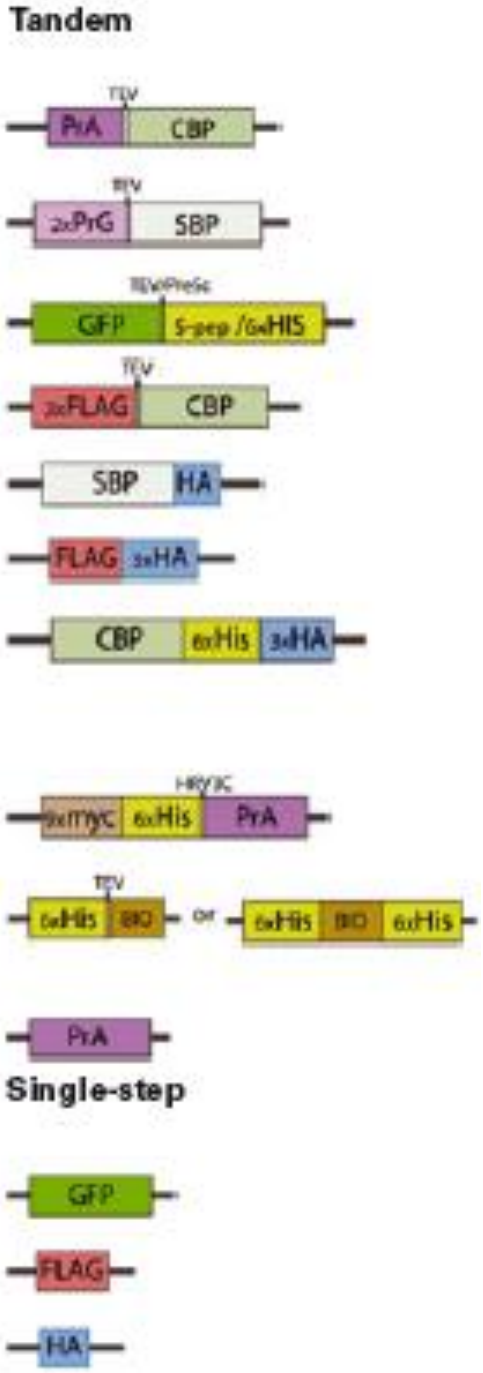
Oeffinger, Proteomics, 2012

MODIFIED TAP tags

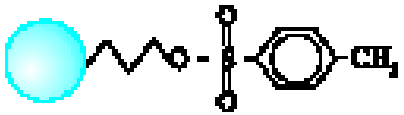
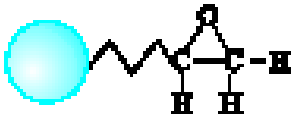
mammalian cells



Drakas et al., Proteomics, 2005
Van Leene et al., TiPlSci, 2008;
Gloeckner et al, Proteomics, 2007
Oeffinger, Proteomics, 2012



MAGNETIC BEADS vs SEPHAROSE

Dynabeads® M-280 Tosylactivated	**Dynabeads® M-270 Epoxy**
	
• Hydrophobic bead. • Surface tosyl groups. • Bead diameter 2.8 µm.	• Hydrophilic bead. • Surface epoxy groups. • Bead diameter 2.8 µm.
• Direct covalent binding to primary amino- or sulfhydryl groups in proteins and peptides. • No further surface activation required. • Binding over night at neutral to high pH and high temperature.	• Direct covalent binding to primary amino and sulfhydryl functional groups in proteins and peptides. • No further surface activation required. • Binding over night at neutral pH, high salt and a wide temperature range.

Diameter 2.8 um volume 40 000 smaller than agarose/sepharose

METHODS TO STUDY TRANSCRIPTOMES

- **SAGE - serial analysis of gene expression**

sequencing of small cDNA tags generated by type II restriction enzymes

- **CAGE - cap analysis of gene expression**

sequencing of small cDNA tags derived from capped transcripts

- **3' long SAGE**

identification of SAGE tags that originate from 3' ends of transcripts

- **RNA Seq - high throughput sequencing of cDNAs**

- **GRO-seq - genomic run-on sequencing**

sequencing of cDNA tags extended from nascent transcripts

- **tiling arrays**

microarrays with overlapping probes that cover the complete genome

METHODS TO STUDY TRANSCRIPTOMES

- **ChIP (ChIP-chip, ChIP-Seq) - chromatin immunoprecipitation**

indirectly reveal unknown ncRNAs

- **RIP-Seq - RNA immunoprecipitation-sequencing**

- **ChIRP – Chromatin isolation by RNA Purification (+RNA-Seq)**

- **ChART - Capture Hybridization Analysis of RNA targets (+RNA-Seq)**

biotinylated oligonucleotides used to enrich for DNA sequences associated with a particular RNA

- **CRAC - CRosslinking and Analysis of cDNA**

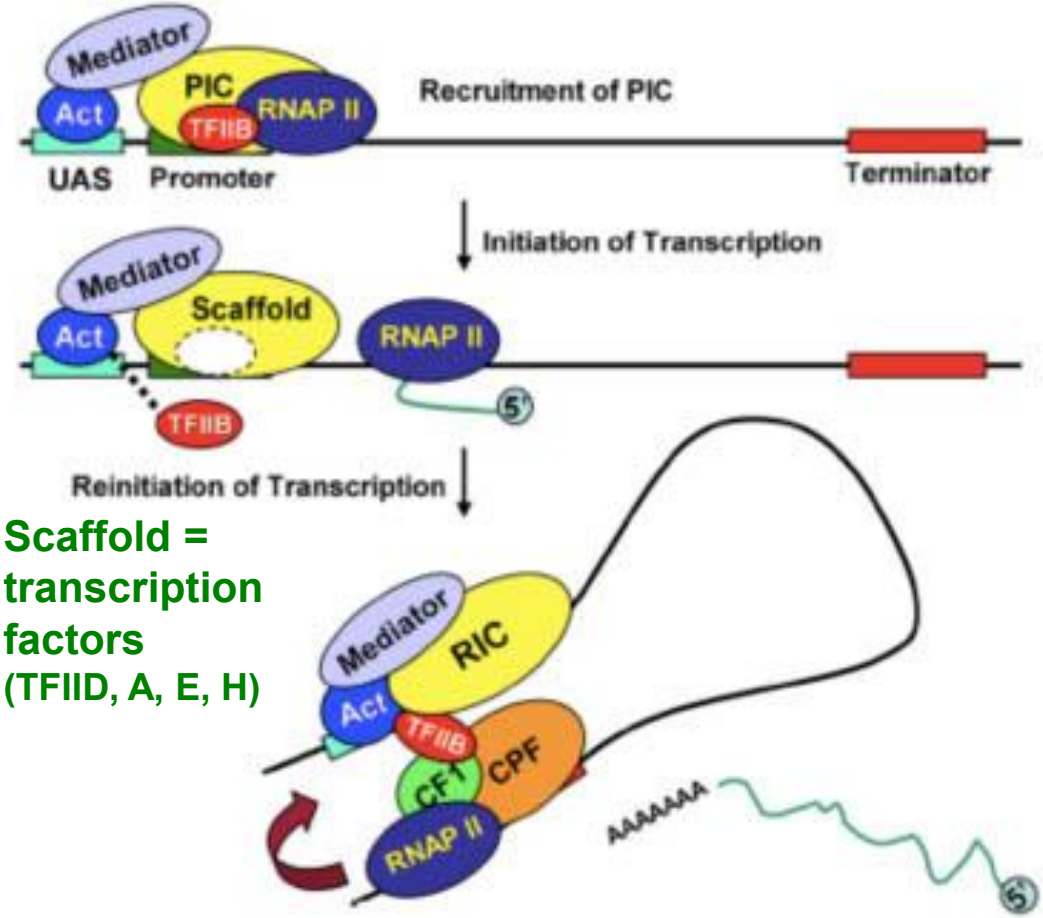
- **PAR-CLIP - PhotoActivatable ribonucleoside–enhanced CrossLinking and ImmunoPrecipitation**

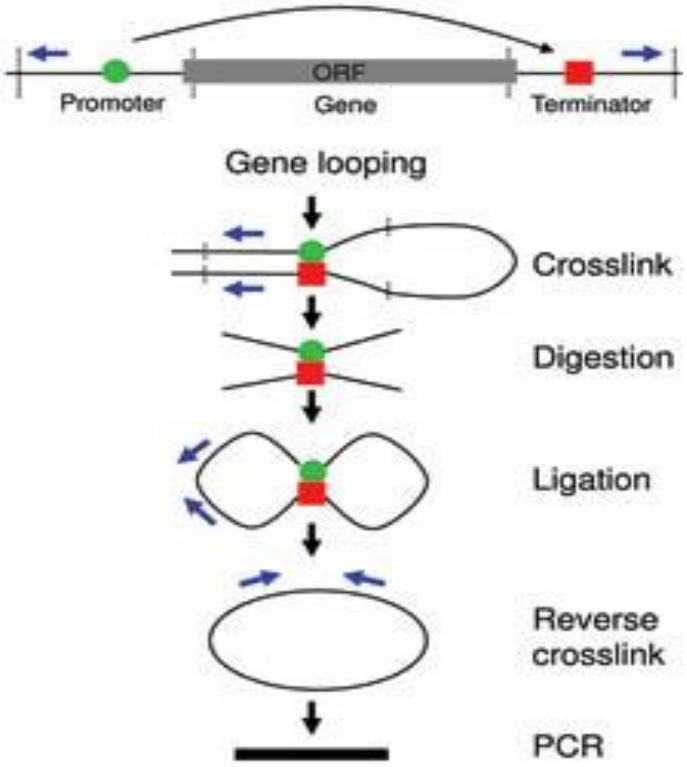
- **HITS-CLIP - High-Throughput Seq CLIP**

GENE LOOPING (long range)

activated transcription

3C chromosome conformation capture





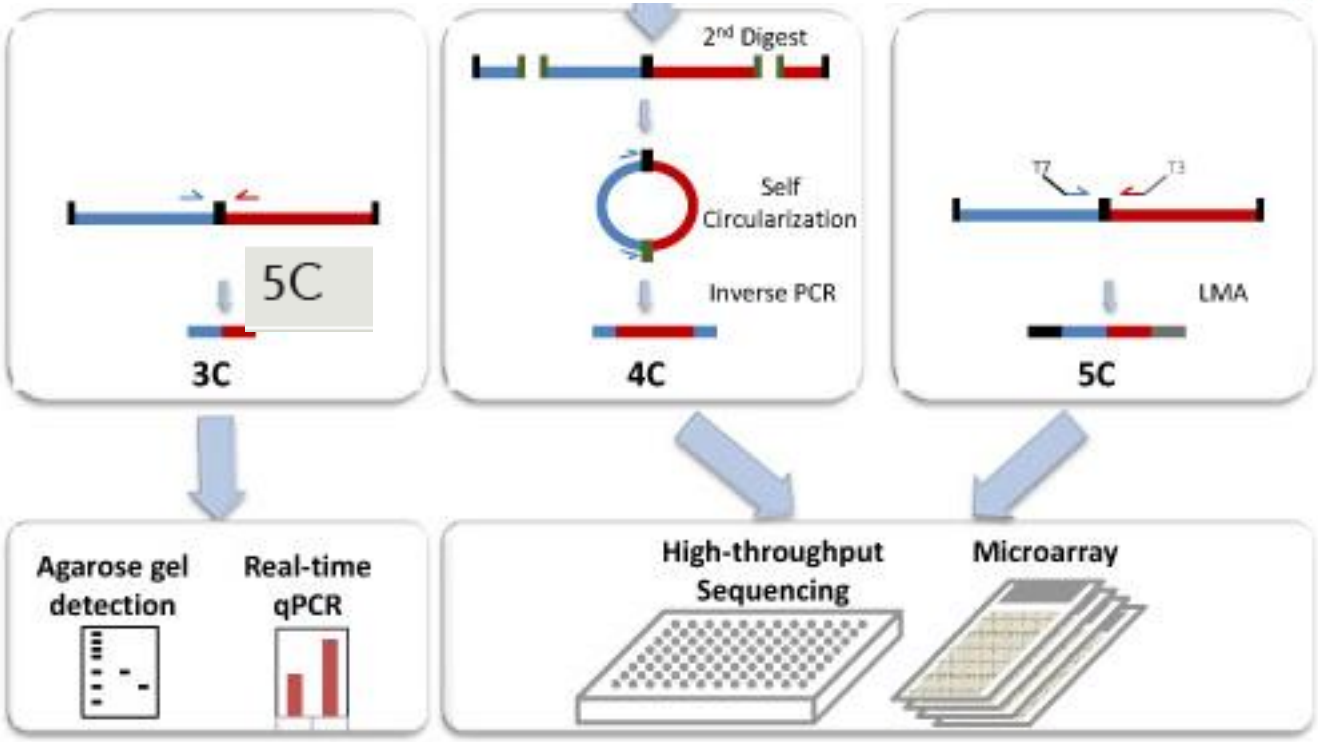
Scaffold = transcription factors (TFIID, A, E, H)

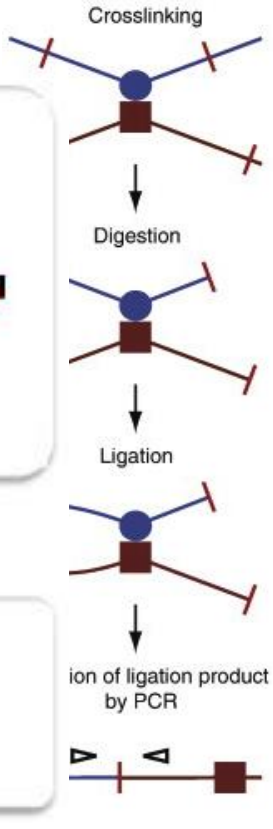
Ansari and Hampsay, GDev, 2005; El Kaderi et al., JBC, 2009

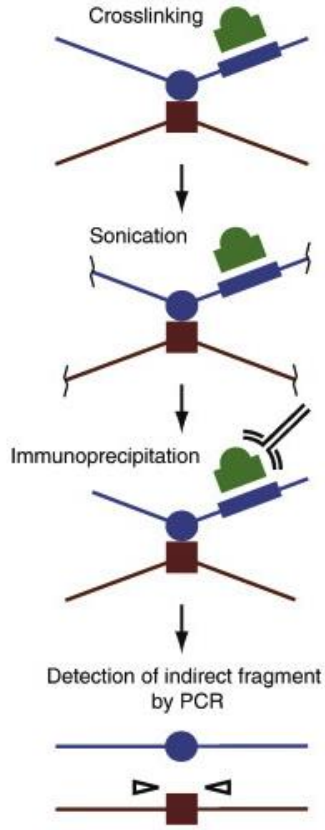
Loop formation requires interaction between factors at the promoter (THIIB) and terminator (Rna15 from CF1) /in mammals: transcription factors, nuclear receptors, insulators, chromatin remodellers, Polycomb, architectural proteins/

Loop function: facilitation of transcription reinitiation of PolII, but also repression of gene expression (PcG, DNA methylation)

3C 4C 5C .....







3C **Chromosome Conformation Capture**
4C **Circularized CCC** (enhanced 3C)
5C **Carbon-Copy CCC** with multiple ligation-mediated amplification (LMA)
GCC **Genome CC** (Hi-C – deep Seq); ChIP version of GCC as 6C
ChIP Loop (indirect ChIP); **6C** **ChIP GCC**

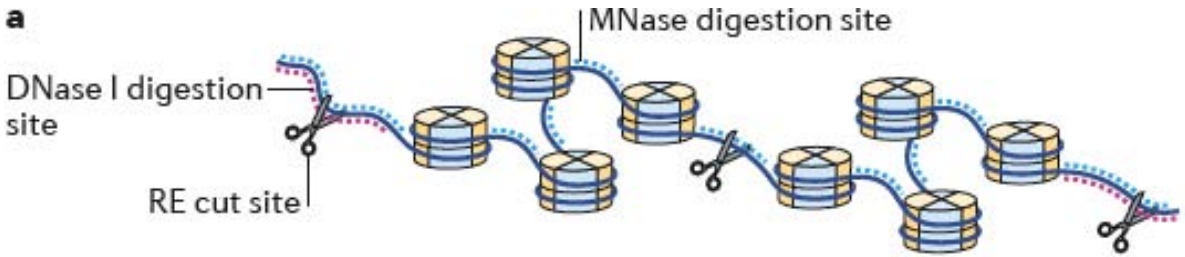
3C **Indirect ChIP**

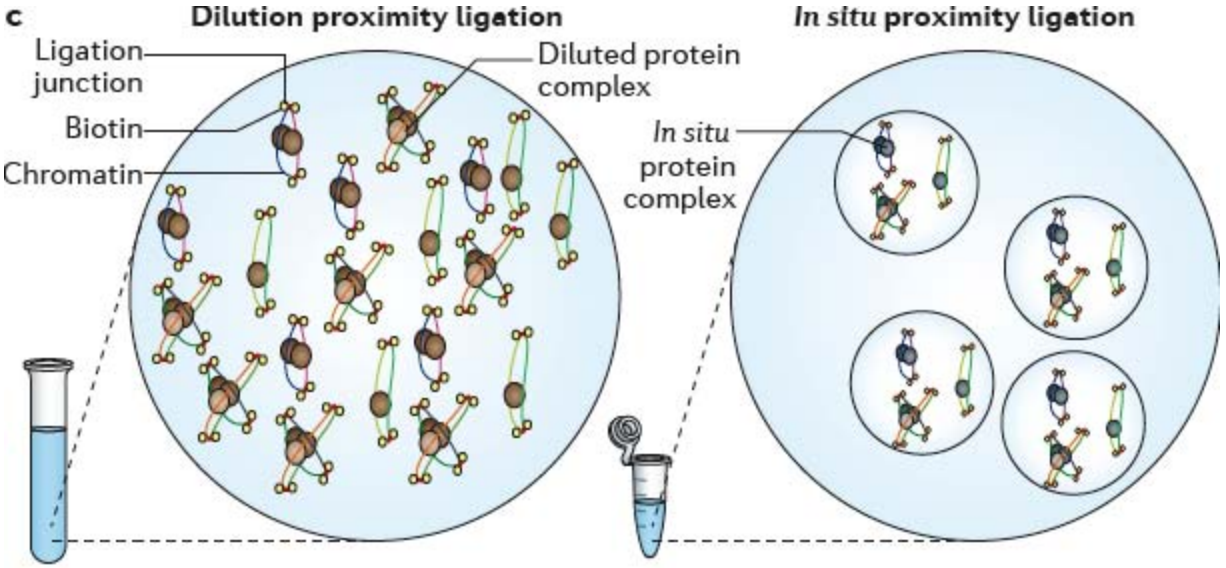
3C | Open-ended 3C | 3C-DSL | e4C | Capture-3C | 3C-seq

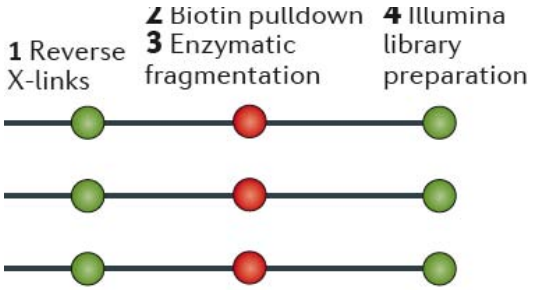
4C | 4C-seq | TLA | e4C | ACT | TCC | GCC | ELP | Hi-C | 5C

ChIA–PET | Capture-HiC | Single-cell Hi-C | *In situ* Hi-C | DNase Hi-C | Micro-C

3C 4C Hi-C





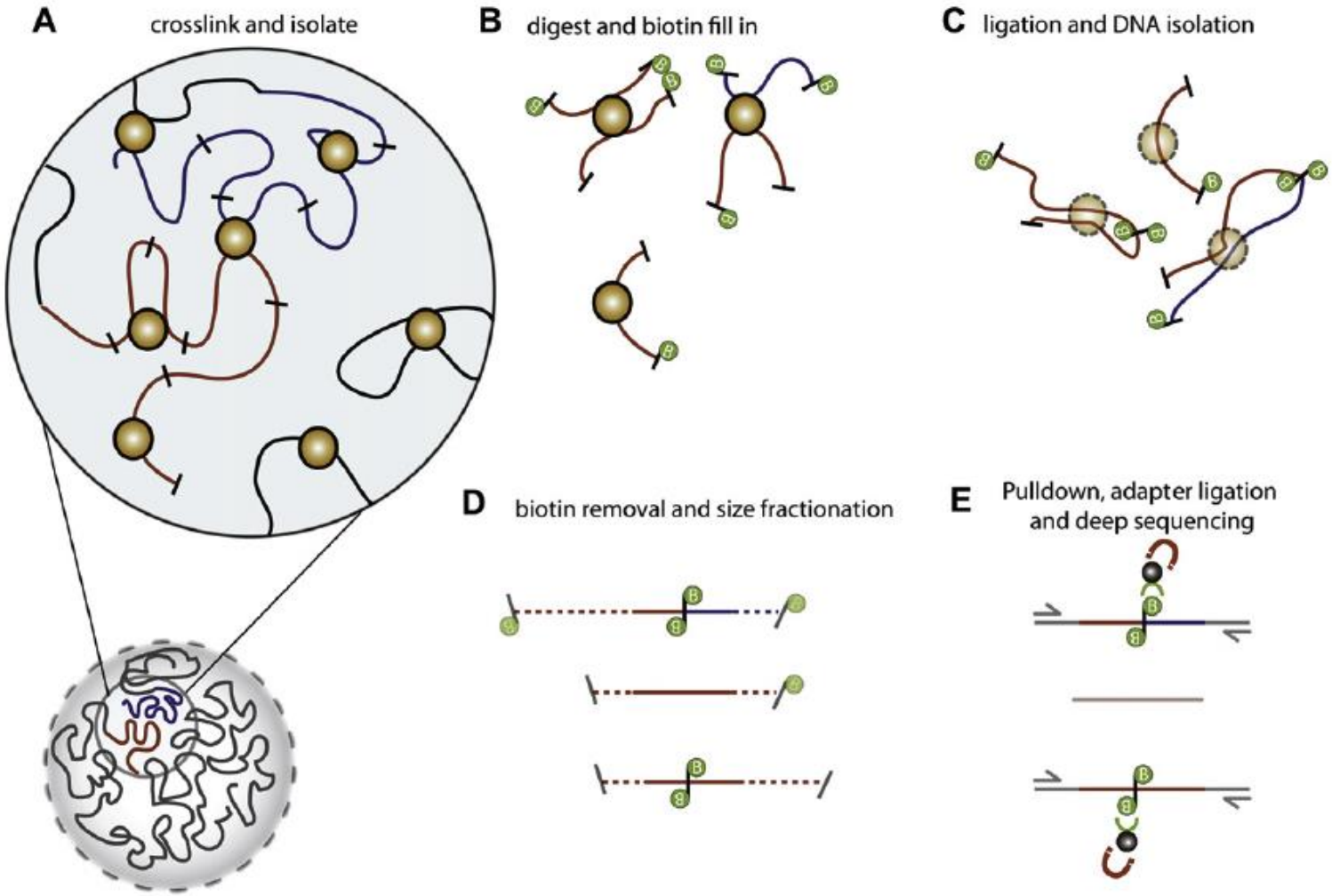


Schmit et al., Nat Rev Mol Cell Biol, 2016

Hi-C

- chromatin crosslinking, digestion, re-ligation, PCR amplification/deep sequencing

- biotin-labeled nucleotide incorporated at the ligation junction for selective purification of chimeric DNA ligation junctions



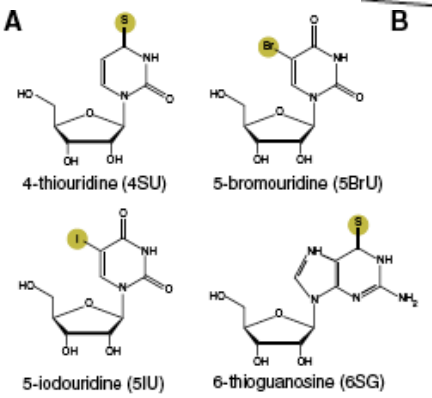
Belton et al., Methods, 2012

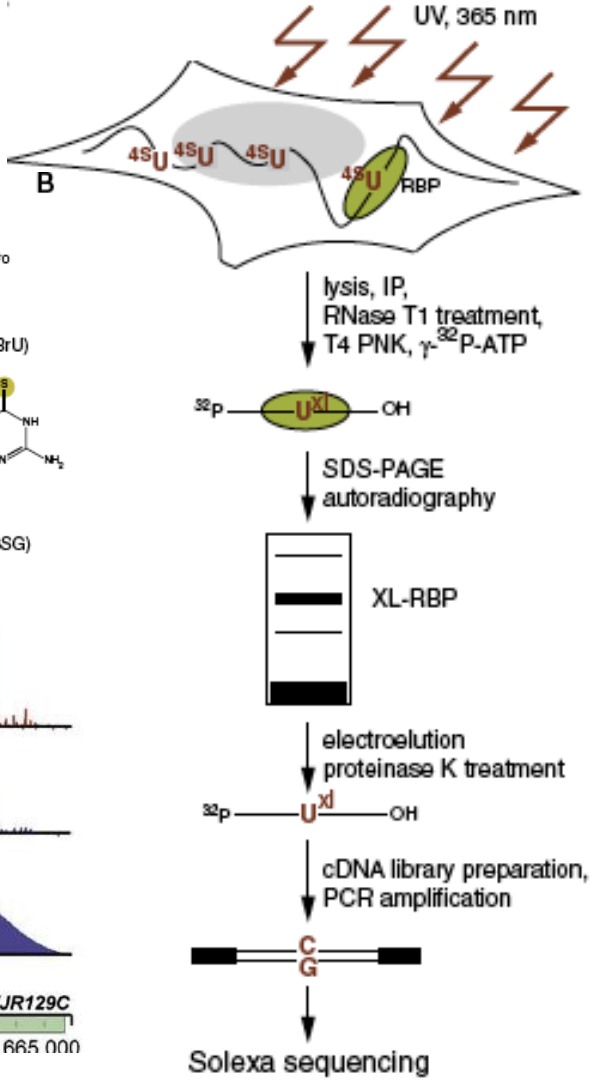
PAR-CLIP

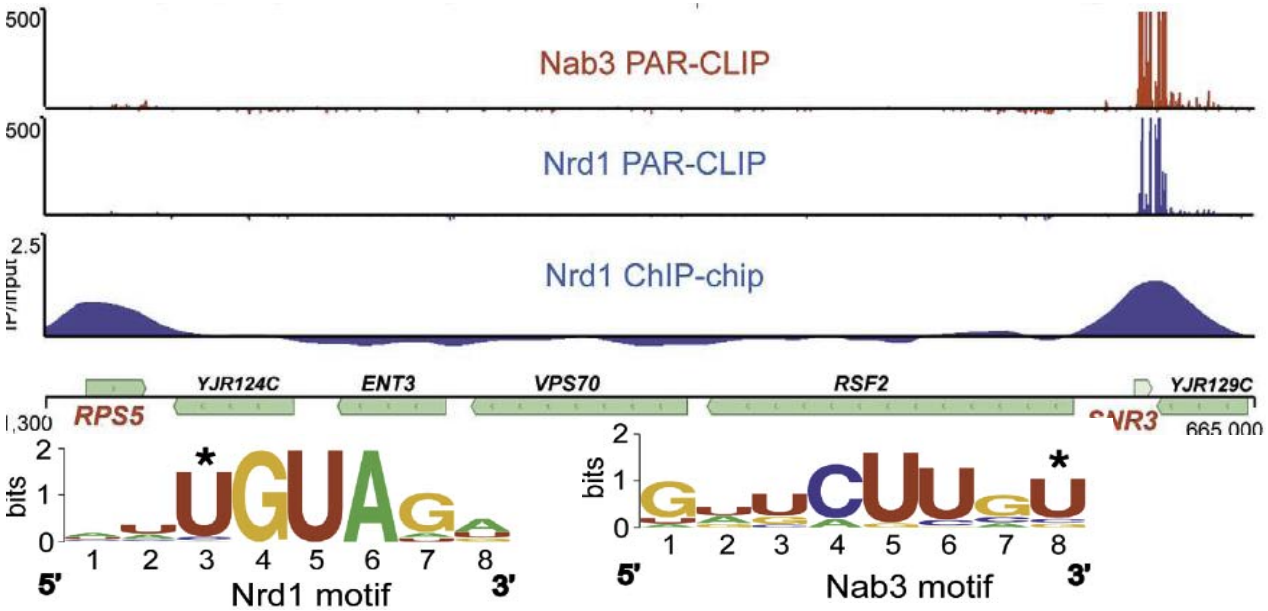
PhotoActivatable ribonucleoside–enhanced CrossLinking and ImmunoPrecipitation

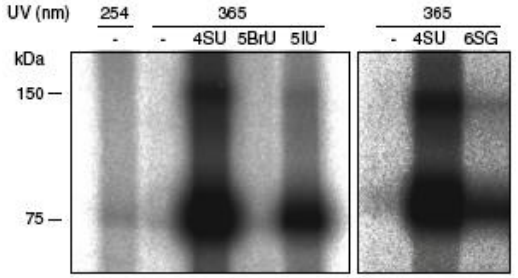
HITS-CLIP:

High-Throughput Seq CLIP





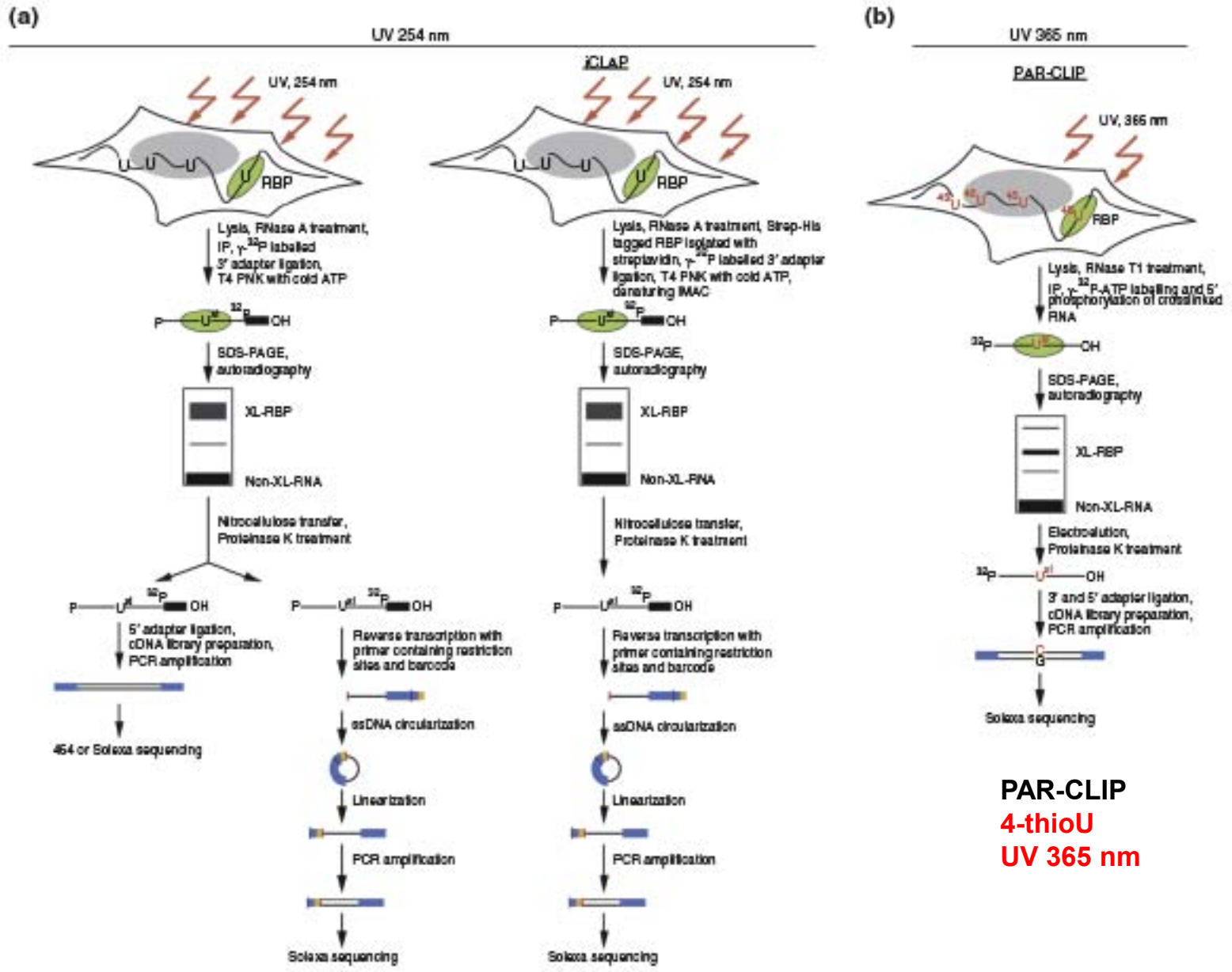




Creamer et al., PLOS Genet, 2011

Hafner et al., Cell, 2010

http://www.jove.com/index/details.stp?ID=2034



PAR-CLIP
4-thioU
UV 365 nm

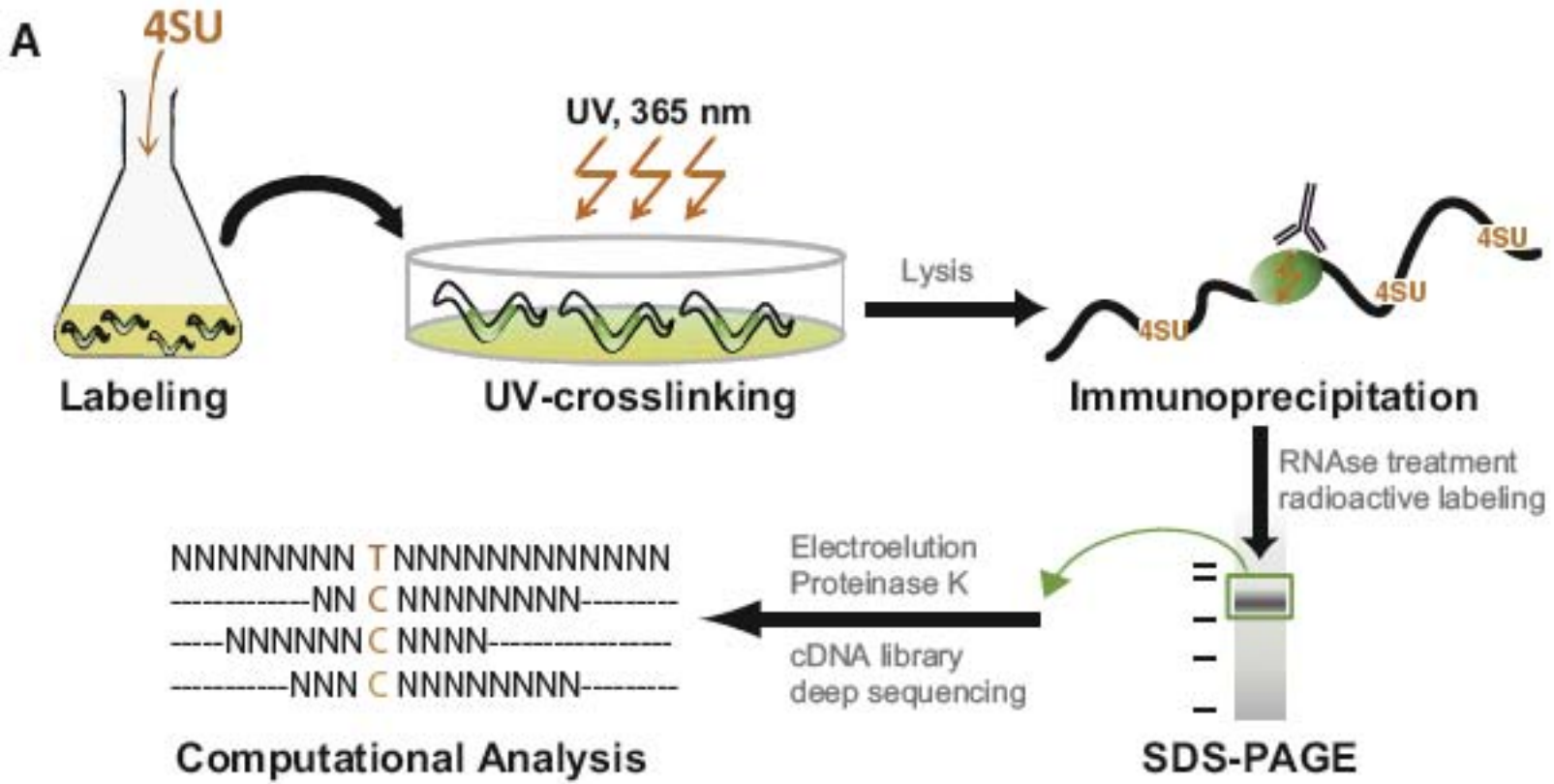
UV 254 nm **HiTS-CLIP**

iCLIP
ssDNA is circularized

iCLAP
RBP is Strep- and polyHis tagged

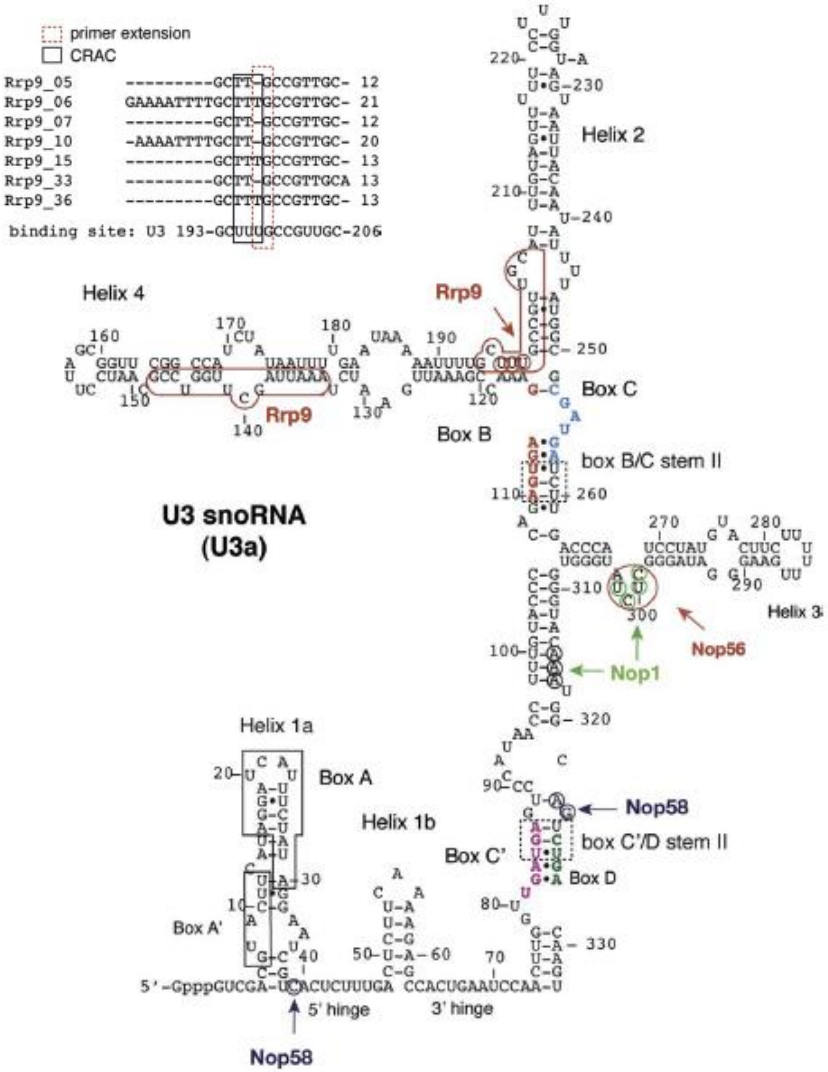
Ascano et al., WIREs RNA, 2012

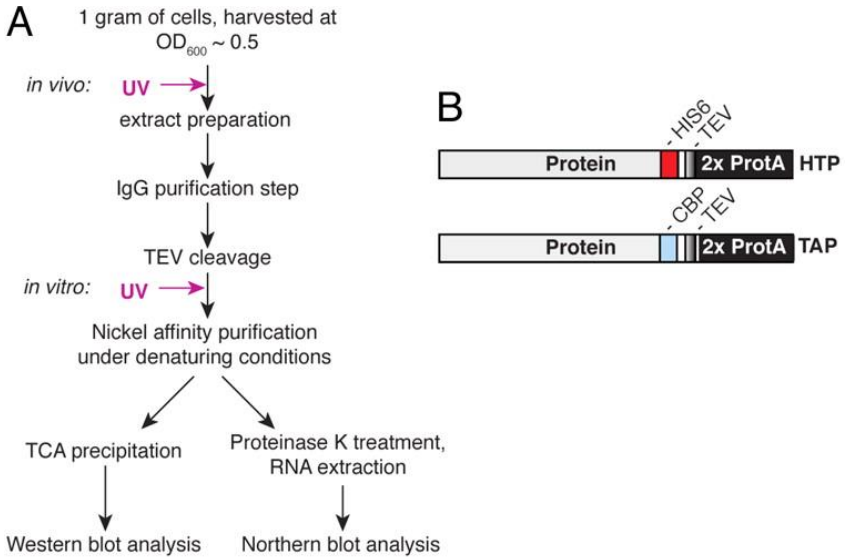
in vivo PAR-CLIP

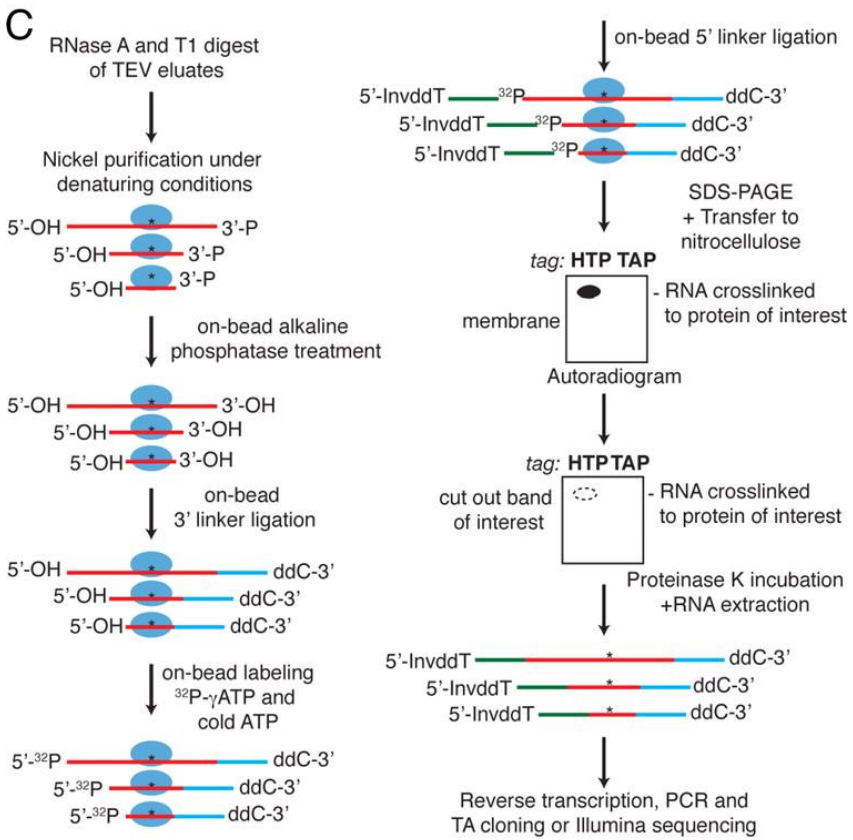


Jungkamp et al., Mol Cell, 2011

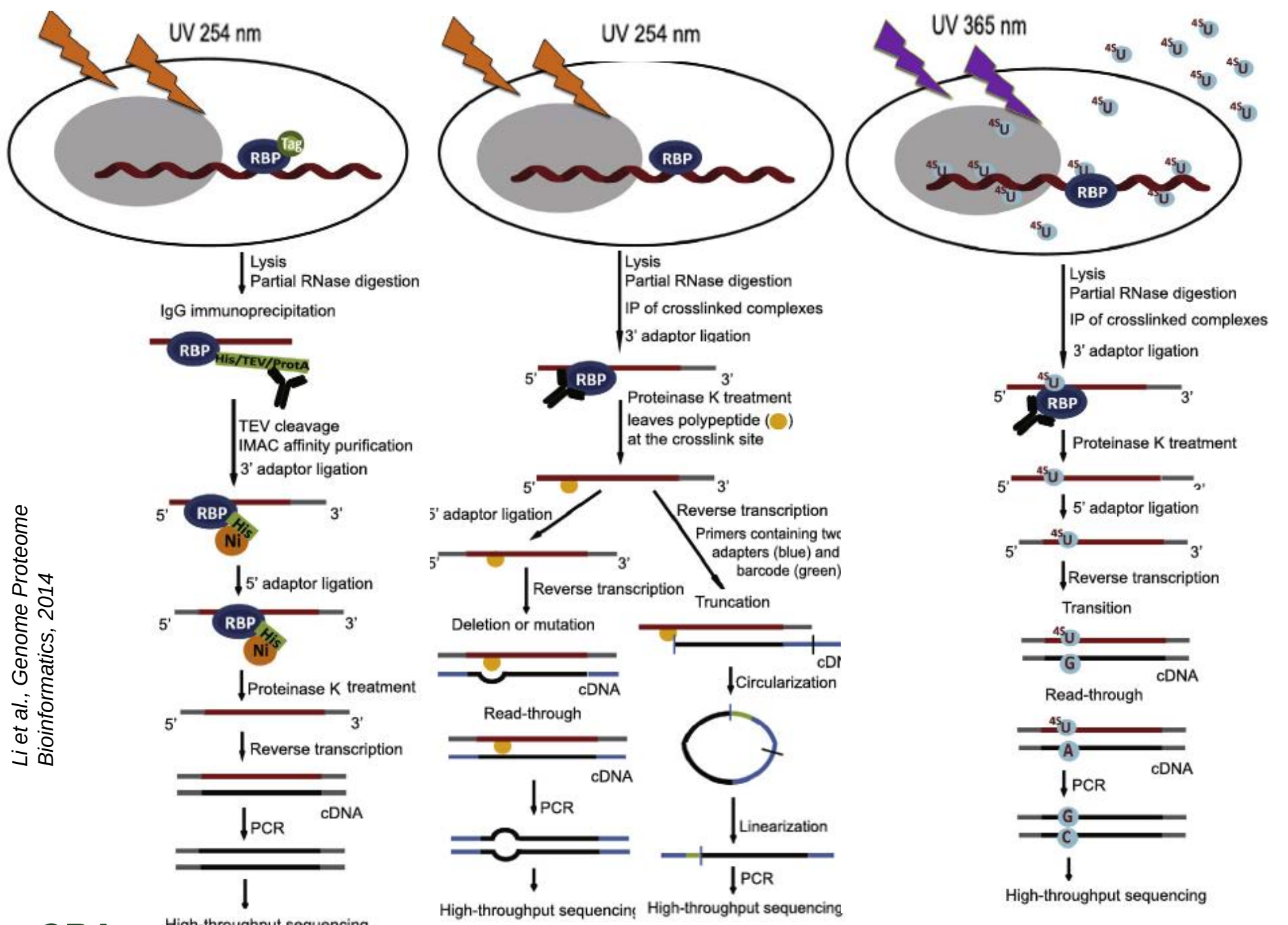
CRAC technique:
CRosslinking and Analysis of cDNA





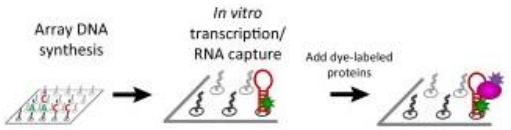
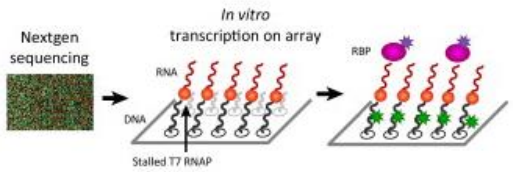
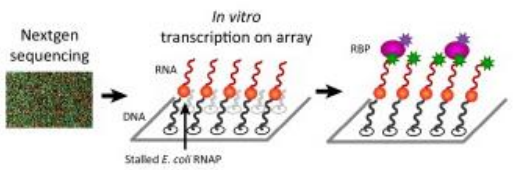
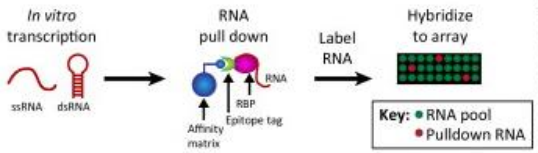
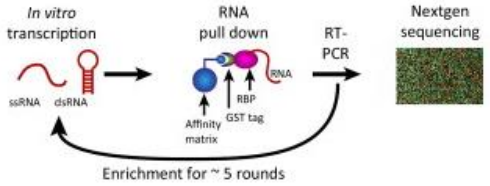
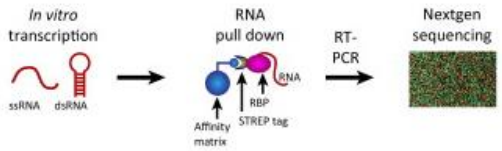


Granneman et al., PNAS, 2009



Li et al., Genome Proteome Bioinformatics, 2014

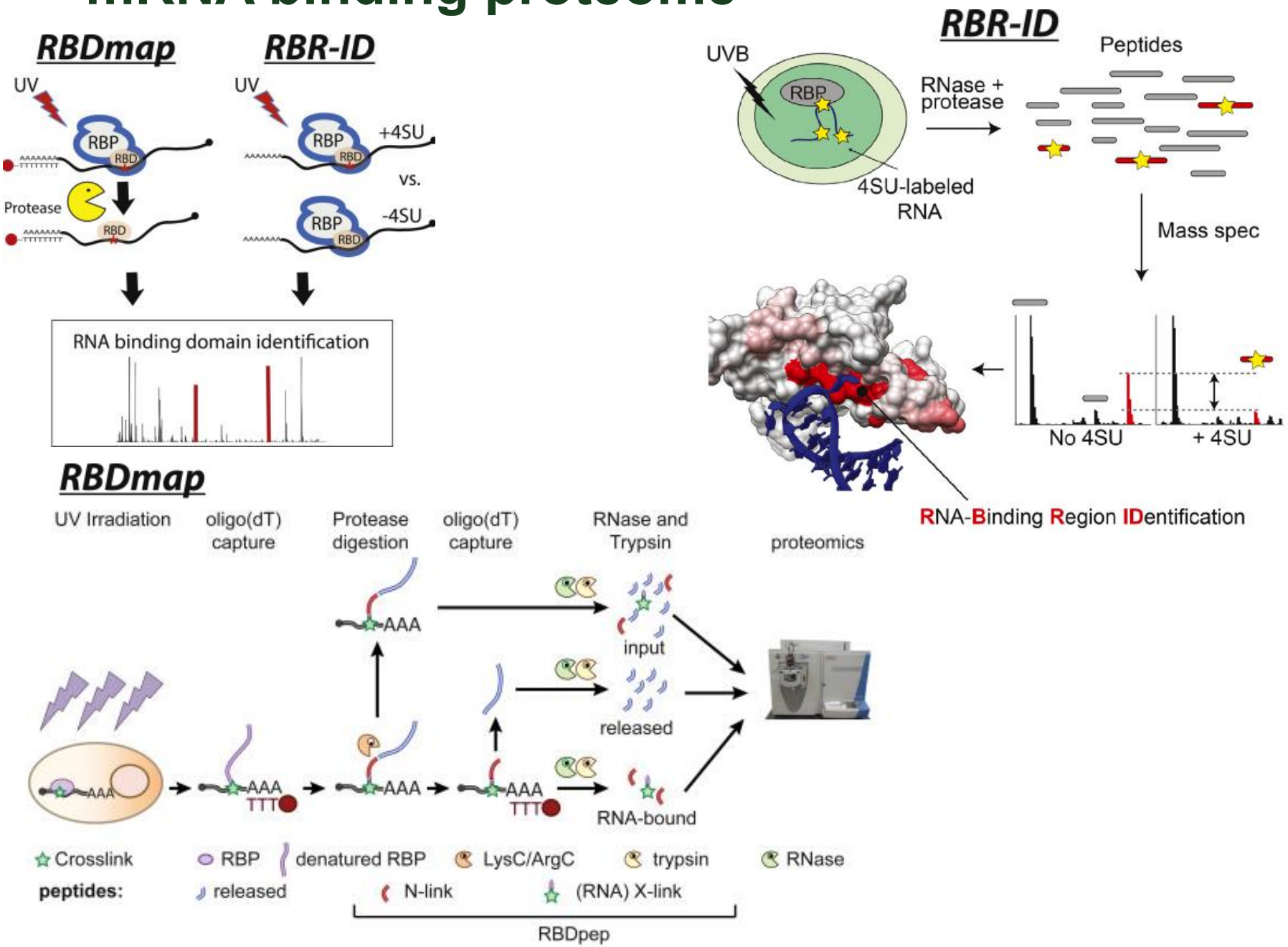
RNA-protein interactions Methods

Name	Molecule detected	Method	# of unique RNA sequences present in input
(A) **RNA-MITOMI**	RNA variants/ protein mutants		384
(B) **HiTS-RAP**	RNA-binding proteins		150,000
(C) **RNA-MaP**	RNA-binding proteins		10,000
(D) **RNA-compete**	RNA-binding proteins		213,000
(E) **SEQRS**	RNA-binding proteins		$1x10^{12}$
(F) **RBNS**	RNA-binding proteins		$1x10^{24*}$

Key: ssRNA, dsRNA, DNA, RNA-binding protein, Bead, Fluorescent label, RNA polymerase

Zachary et al., TiBS, 2015

mRNA binding proteome



Castello et al.; He et al., Cell, 2016

TABLE 2 | Large-Scale Methods for the Discovery of RBP/RNP RNA Targets

Method	Goal	Description	Selection of RBPs Analyzed
Non-crosslinking methods			
RIP-Chip	Target gene identification	IP of epitope tagged or endogenous RBP/RNP to isolate-associated RNAs. Isolated RNAs are analyzed by microarray. RBP/RNP targets are defined by calculating and scoring according to enrichment of IP'd transcripts over control expression values.	For comprehensive list, see Table 1 in Ref 33.
RNA IP and high throughput sequencing (RIP-Seq)	Target gene identification	Same procedure as RNA IP followed by microarray analysis (RIP-Chip); IP'd RNA is quantified by RNAseq. RBP/RNP targets are defined, similar to RIP-Chip, by calculating enrichment scores over control.	TDP-43,[34] LIN-28,[35] Polycomb proteins[32]
Photocrosslinking methods			
CLIP	Target site identification; definition of RRE	Ultraviolet (UV) 254 photocrosslinking of RNA to RBPs in live cells or tissues prior to lysis. Crosslinked RNA is trimmed by RNases and RNA–protein complexes are fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Crosslinked RNAs are isolated, 3′ and 5′ adapters for RT and polymerized chain reaction ligated. The cDNA library is inserted into plasmid for bacterial transformation, cloning, and sequencing.	Nova,[36] SF2,[37] CUGBP1,[38] Rsr,[39] MSY2,[40] SAM68,[41] hnRNP A1[42]
HITS-CLIP	Target site identification; definition of RRE	Similar to standard CLIP, however, cDNAs are deep sequenced (454 or Solexa). Overlapping sequence reads are clustered and RBP/RNP targets are defined based on enrichment over negative controls.	TDP-43,[43,44] Nova,[45] AGO2,[46,47] Alg-1,[48] SFRS1,[49] FOX2,[50] PTB,[51] Khd1[52]
iCLIP	Target site identification; definition of RRE	Crosslinking procedures similar to CLIP. Partial cDNAs, generated from stalled RT of crosslinked RNAs, are circularized and ultimately deep sequenced. RBP/RNP targets are similarly defined as in HITS-CLIP. Sites of crosslinking are interpreted as the (−1) position a sequence read maps to (indicating the putative RT stall sites).	hnRNP C,[53] TIA1, TIAL1[54]
iCLAP	Target site identification; definition of RRE	Similar to iCLIP except that the RBP is Strep- and polyhistidine epitope tagged. Streptavidin beads are used in the first purification step. Immobilized metal-ion affinity chromatography (IMAC) under denaturing conditions is performed as a secondary purification. Isolated RNAs are converted into cDNA and sequenced similar to iCLIP.	TIA1, TIAL1
CRAC	Target site identification	Similar in concept to CLAP, where a tandem affinity purification protocol is used. RBPs are engineered to contain C-terminal 6X-histidine, tobacco etch virus (TEV) protease site, and Protein A tags. Immunoglobulin G beads are used as a first purification step, followed by TEV protease treatment to elute crosslinked RNA–protein complexes. IMAC is performed under denaturing conditions as a secondary purification. Individual RNAs were analyzed by northern blot or sequenced after amplification with gene-specific primers.	Prp43,[55] U3 snoRNA-binding sites of Nop1, Nop56, Nop58, and Rrp9[56]
Photoactivatable ribonucleoside-enhanced CLIP	Target site identification; definition of RRE	Incubation of live cells with photoactivatable ribonucleosides (4-thiouridine and 6-thioguanosine) that are incorporated into nascent transcripts. UV-365 photocrosslinking of RNA to proteins prior to lysis. IP of epitope tagged or endogenous RBP/RNP to isolate-associated RNAs. cDNA library preparation of isolated RNAs. Putative RBP/RNP target sites are scored by frequency of crosslinking evidence, seen as a characteristic T2C (or G2A) nucleotide mutation.	ELAVL1,[57,58] QKI,[59] IGF2BP1–3,[59] AGO1-4,[59] PUM2,[59] TNRC6A-C,[59] FUS,[60] EWSR1,[60] TAF15[60] In analysis: BICC1, CUGBP1, DHX9, DICER, DND1, EXPORTIN5, FMR1, FXR1, FXR2, ELAVL2–4, LIN28B, MBNL1, MOV10, NCL, P54, RBM20, RBPMS, SND1, TARBP2, TDP-43, TIA1, TSN, TSNAX, TTP, WT1

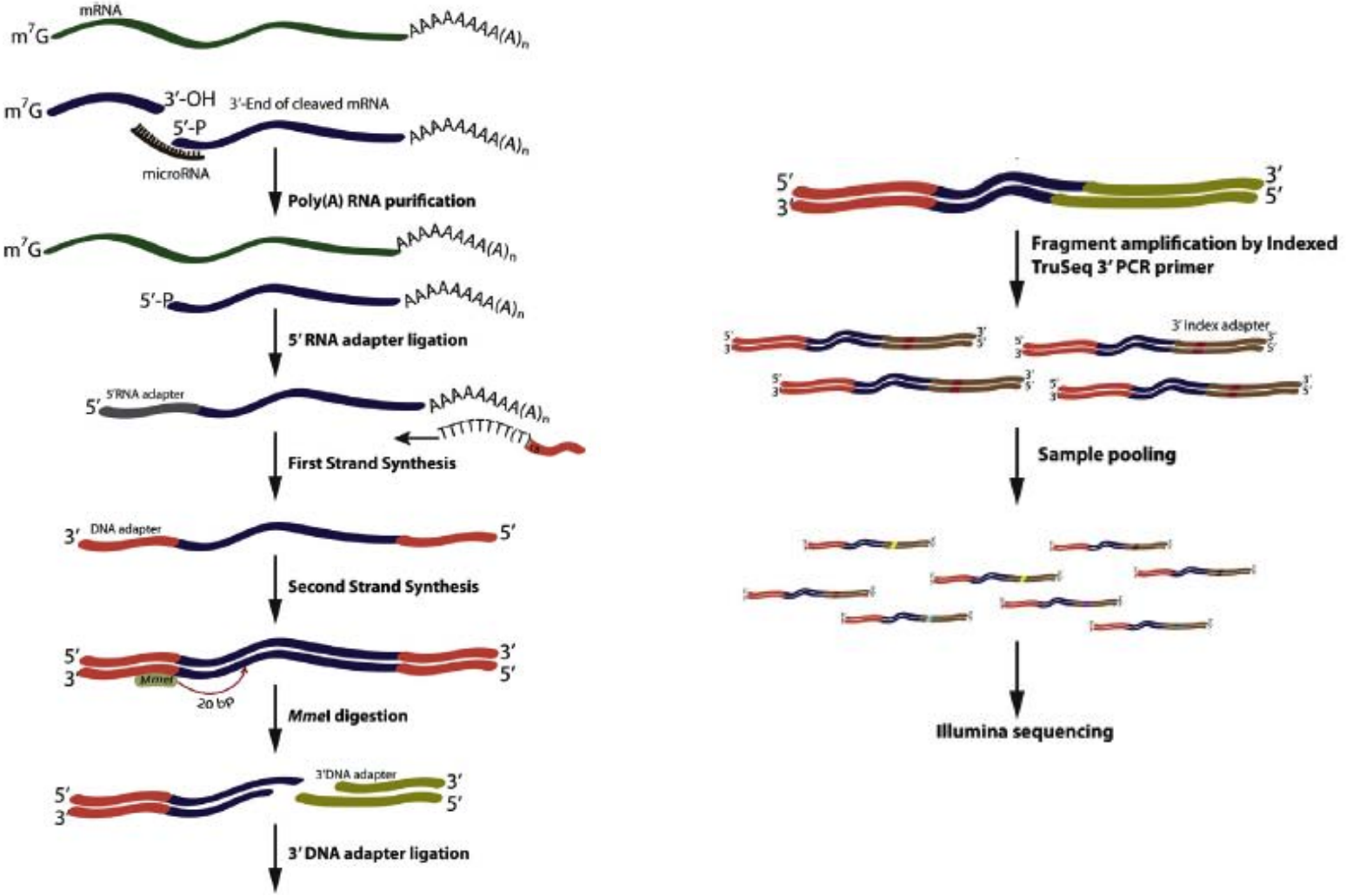
Ascano et al., WIREs RNA, 2012

TABLE 1 | RNA Binding Proteins Studied by CLIP

RNABP	Method	Tissue/Cell Type	Principle Findings	References
Nova	CLIP and HITS-CLIP	Mouse brain	Binding motif	Ule et al.[37]
			Biologically coherent targets	Licatalosi et al.[48]
			RNA splicing map	Racca et al.[16]
			Regulation of poly(A) usage	
			3′ UTR localization	Yano et al.[100]
			Meta analysis elements	Zhang et al.[49]
PTB	HITS-CLIP	HeLa cells	Binding motif	Xue et al.[50]
			RNA splicing map	
Tagged-Khd1	HITS-CLIP	*Saccharomyces cerevisiae*	Binding motif	Wolf and Fink[51]
			Biologically coherent targets	
			Discovery of role in translational regulation	
hnRNP A1	CLIP	HeLa cells	Discovery of role in regulating pri-mRNA processing	Guil and Caceres[52]
Fox2	HITS-CLIP	Human embryonic stem cells	Biologically coherent targets	Yeo et al.[53]
			RNA splicing map	
SFRS1	CLIP and HITS-CLIP	HEK293T cells	Binding motif	Sanford et al.[54]
			Sites relevant to disease	Sanford et al.[55]
Tagged-Rrm4	CLIP	Filamentous fungus *Ustilago maydis*	Binding motif	Becht et al.[56]
			Localization of rrm4-RNPs	
CUGBP1	CLIP	Mouse hindbrain	Binding motif	Daughters et al.[57]
			Validation of splicing target	
Tagged-snRNPs	HITS-CLIP	*S. cerevisiae*	Detailed mapping of protein-rRNA and snoRNA interaction sites	Granneman et al.[58] Bohnsack et al.[59]
Ro homolog Rsr	CLIP	Eubacterium *Deinococcus radiodurnas*	Discovery of interaction with rRNA	Wurtmann and Wolin[60]
hnRNP C	HITS-CLIP	HeLa cells	Binding motif	Konig et al.[61]
			RNA splicing map	
			Discovery of hnRNP particle organization related to splicing	
Ago	HITS-CLIP	Mouse brain; HeLa	Ago-miRNA binding sites defined	Chi et al.[62]
Tagged-Ago	PAR-CLIP	HEK293 cells		Hafner et al.[43]
Alg-1	HITS-CLIP	*C. elegans*	Alg-1-miRNA binding sites defined	Zisoulis et al.[63]
Msy2	CLIP	Mouse seminiferous tubules	Discovery of interaction with piRNAs, other small RNAs	Xu et al.[64]

Darnell, WIREsRNA, 2010

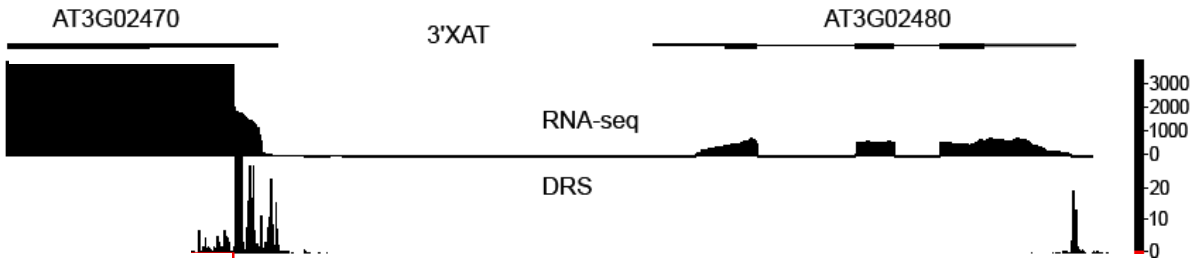
PARE: Parallel Analysis of RNA End
mRNA DEGRADOME RNA-seq

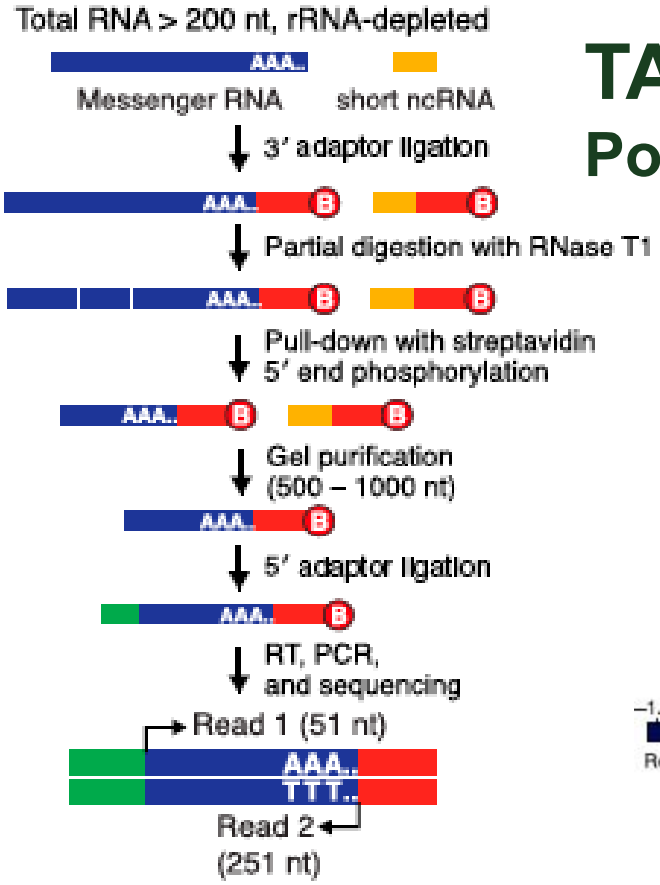


Zhai et al., Methods, 2014

DRS: Direct RNA sequencing

Poly(A) sites

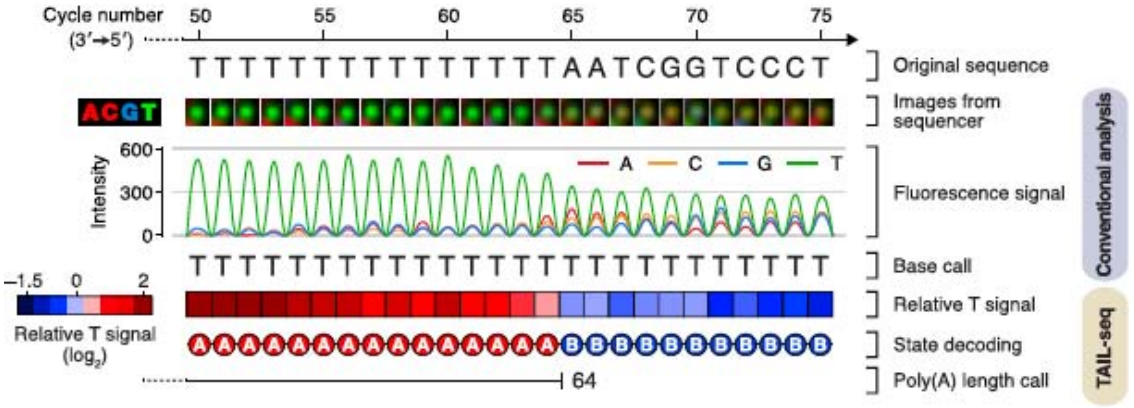




TAIL-seq: RNA 3' end sequencing

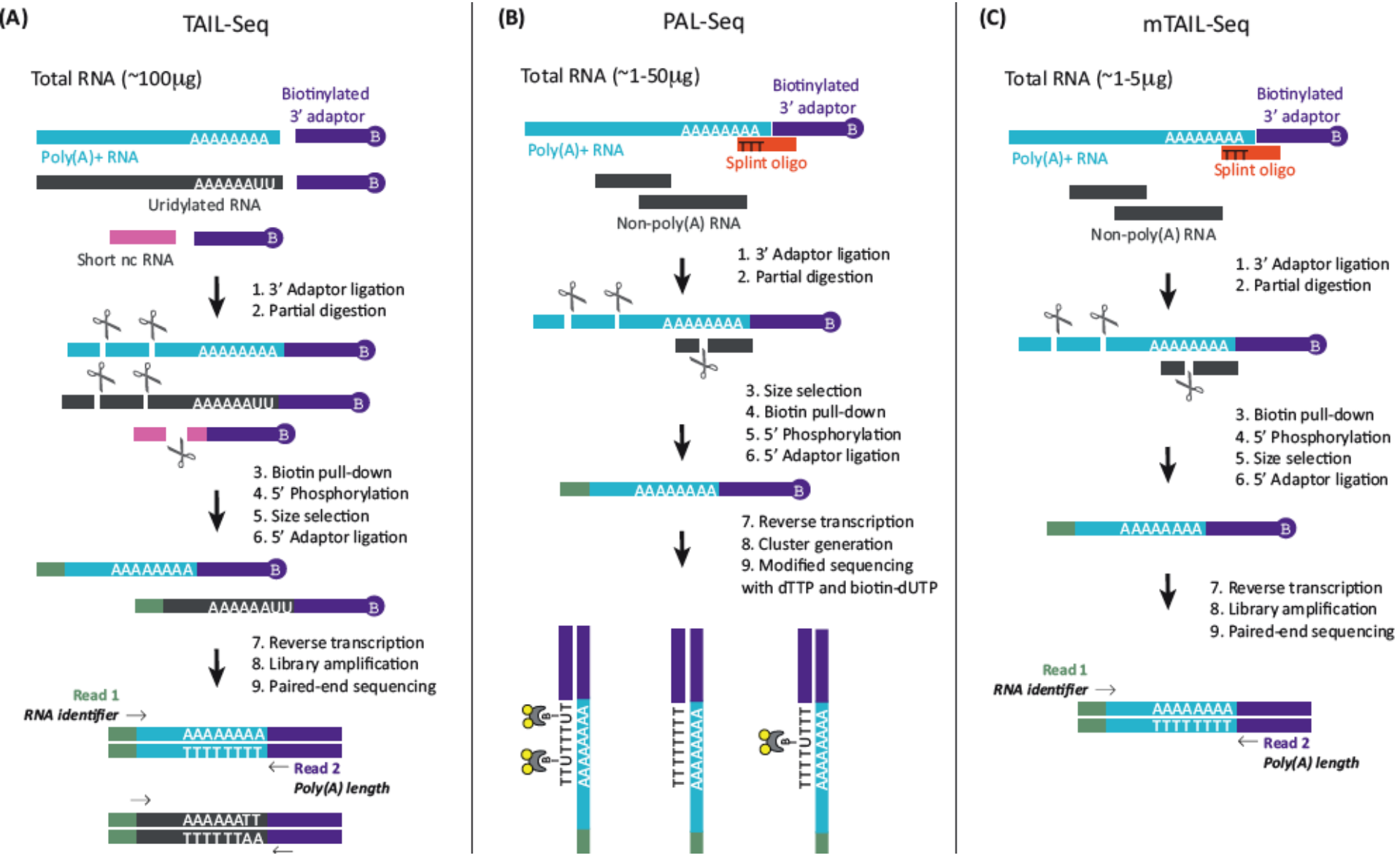
Poly(A) tail length and 3' end modifications

(e.g. U-tailing)



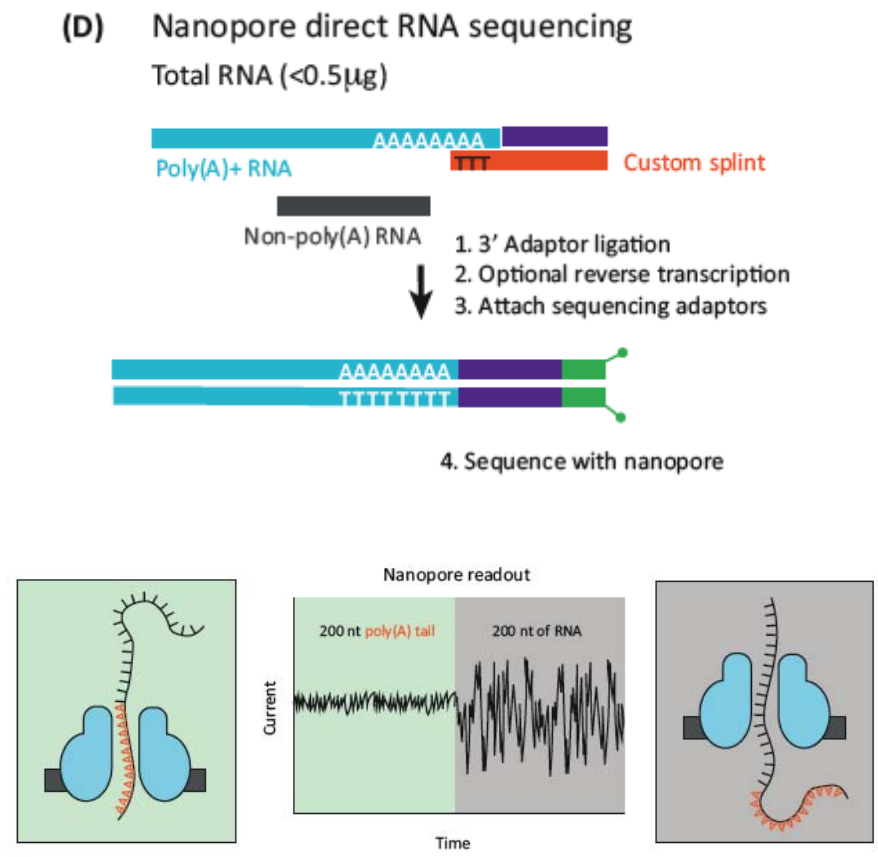
Sherstnev et al., Nat Str Mol Biol, 2012; Zhai et al., Mol Cell, 2014

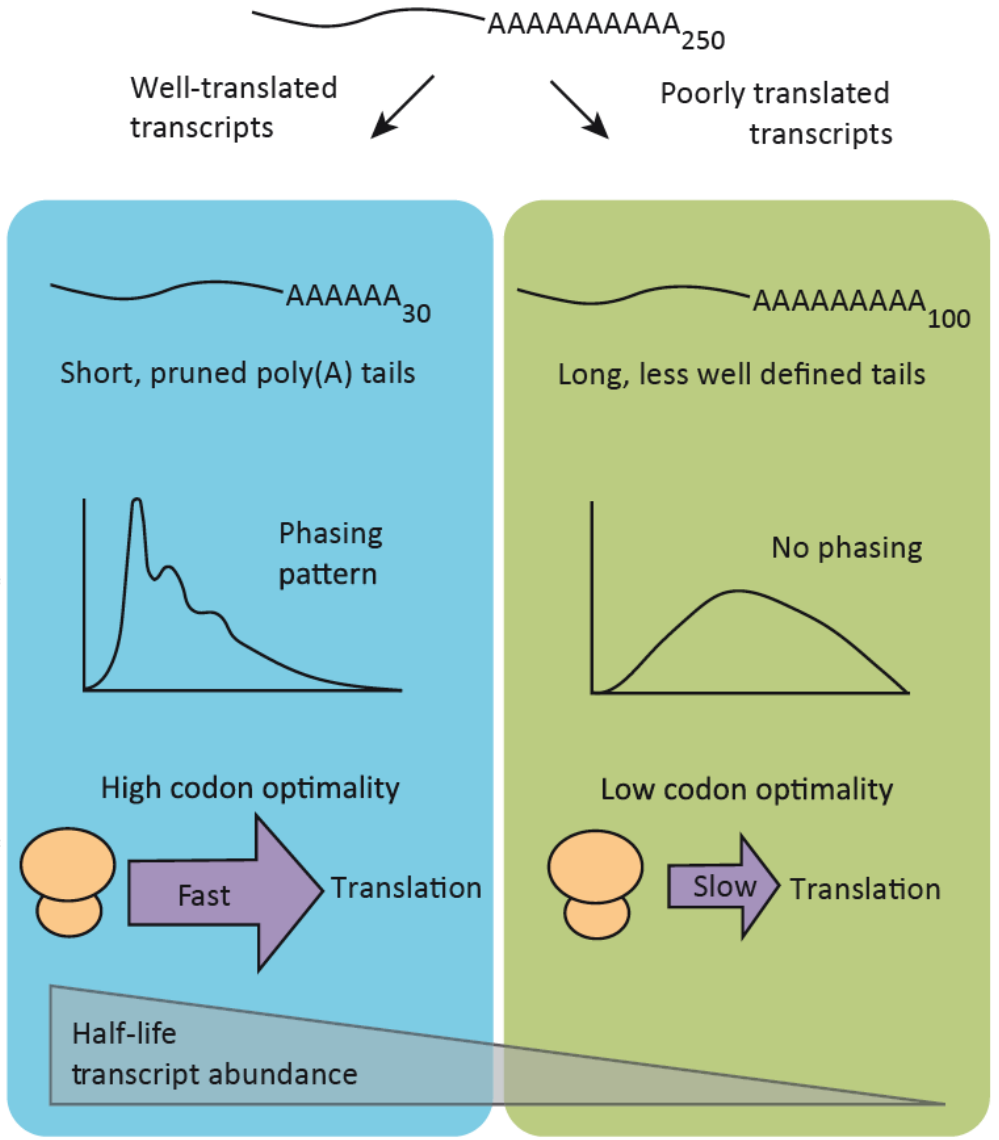
Poly(A) tail analyses



Nicholson and Pasquinelli TiCB 2018

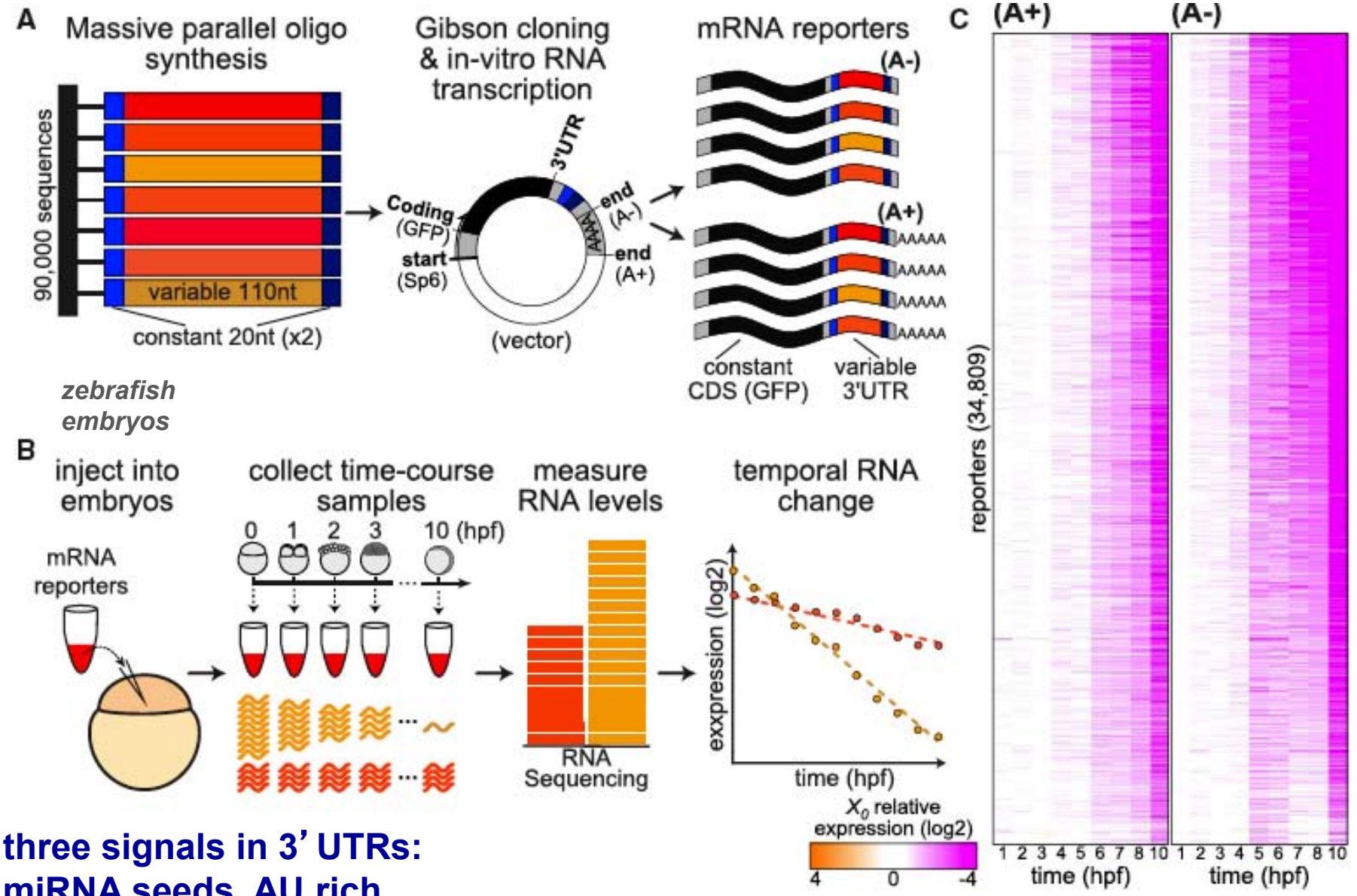
Poly(A) tail analyses





Nicholson and Pasquinelli TiCB 2018

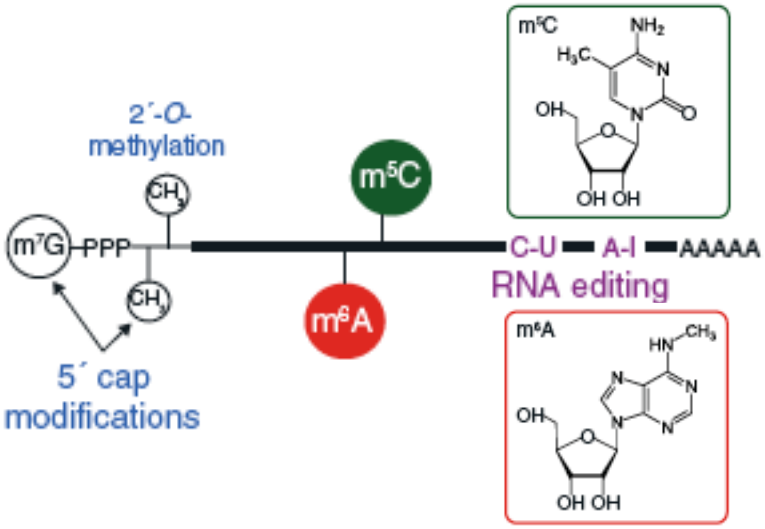
3’ UTR-seq: in vivo analysis of mRNA decay rules

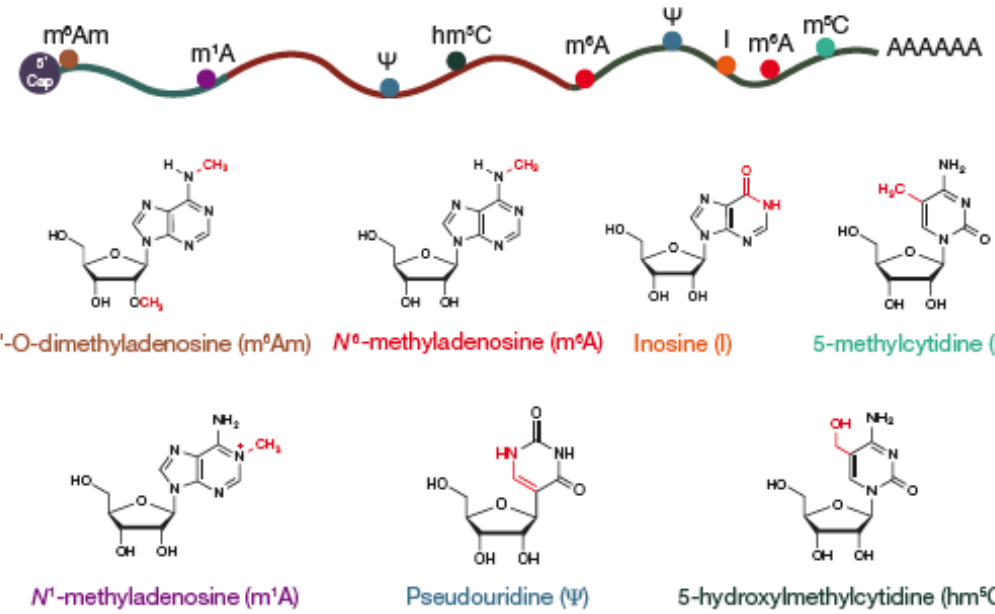


three signals in 3’ UTRs:
miRNA seeds, AU rich,
Pumilio binding sites

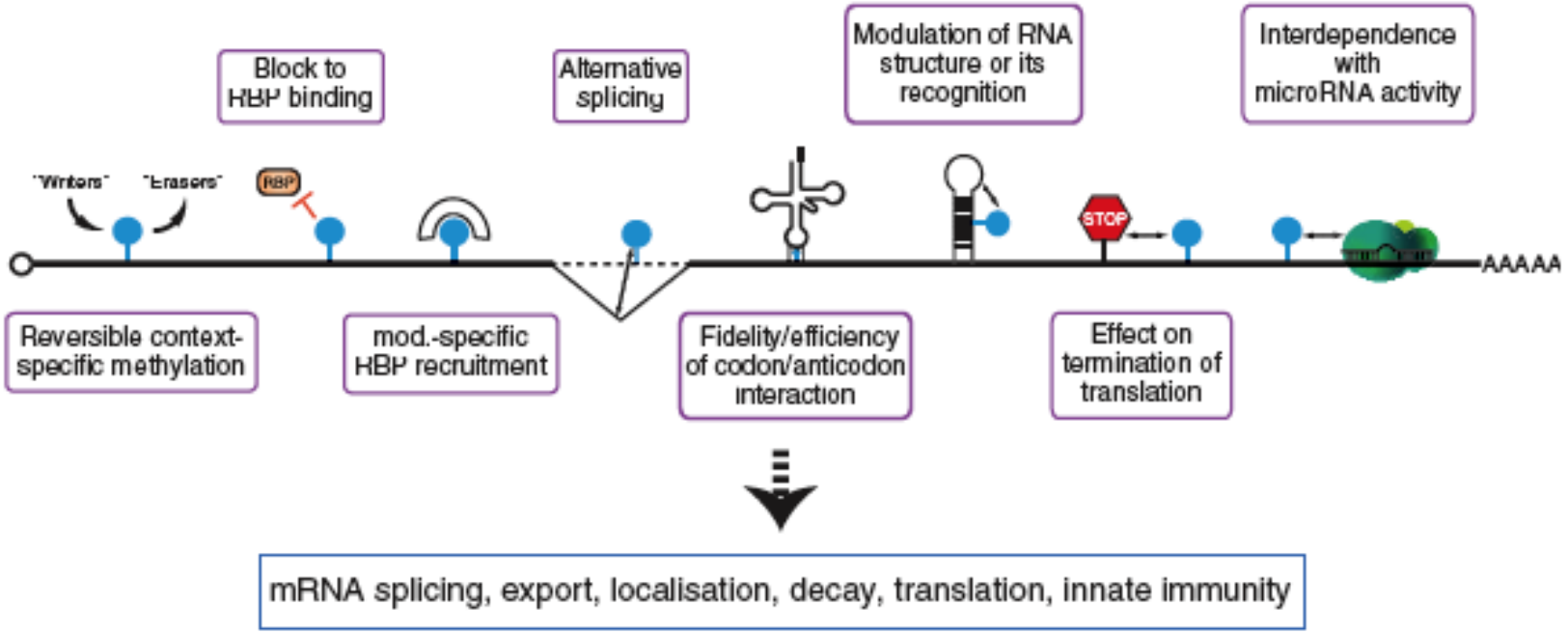
Rabani et al., Mol Cell, 2017

RNA MODIFICATIONS



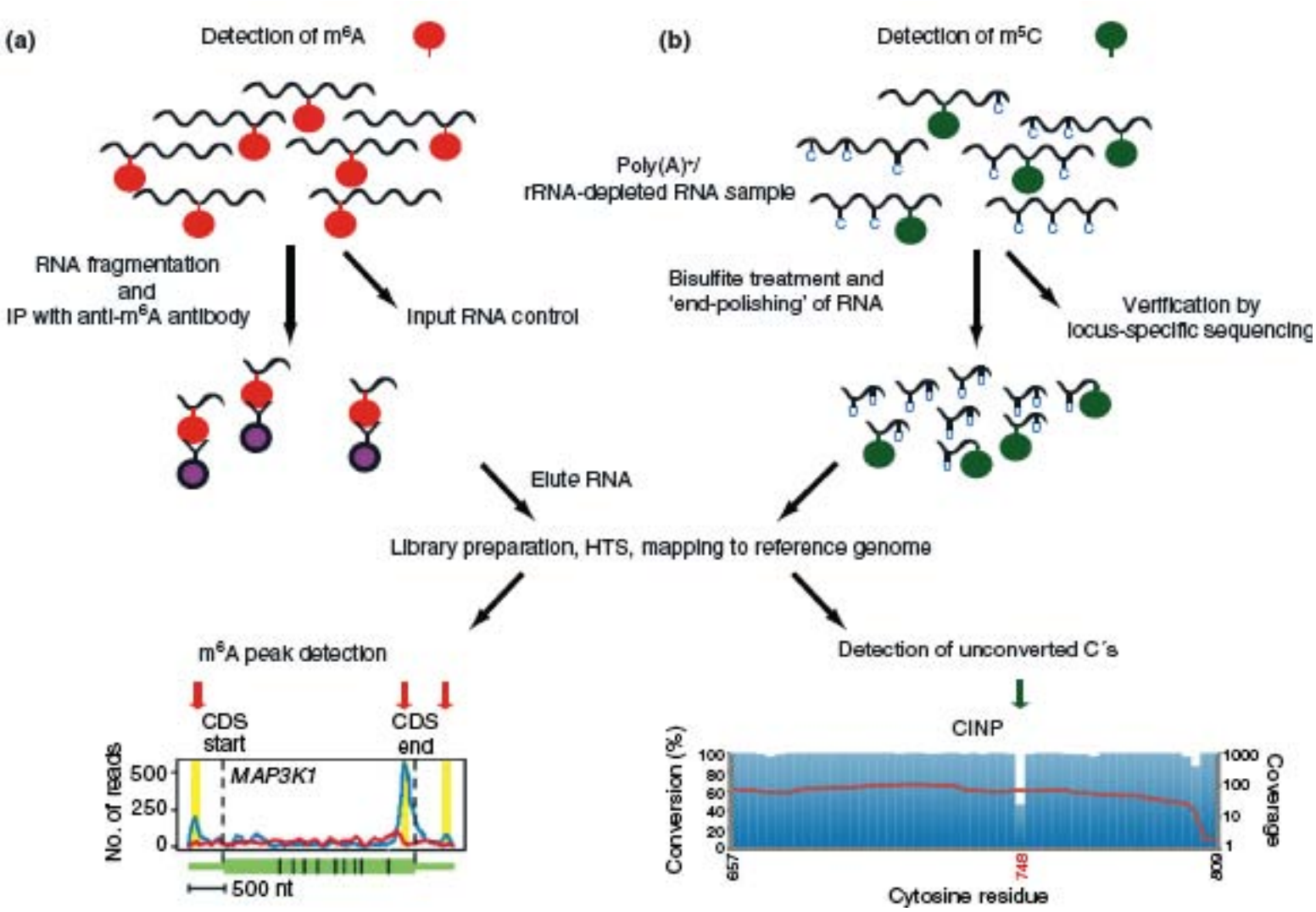


FUNCTIONS



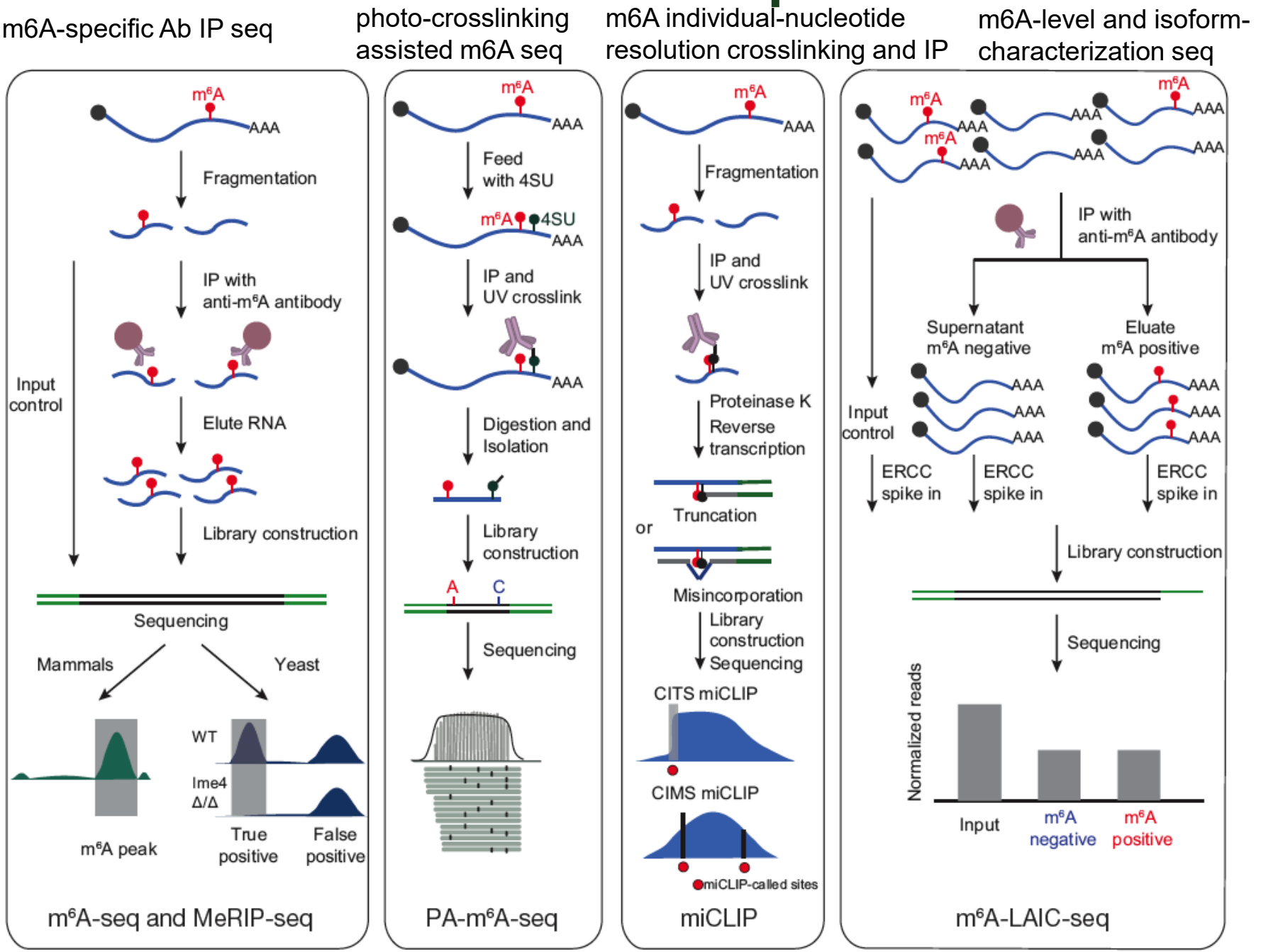
Sibbrit et al, WIREsRNA 2013

RNA MODIFICATION



Sibbrrit et al, WIREsRNA 2013

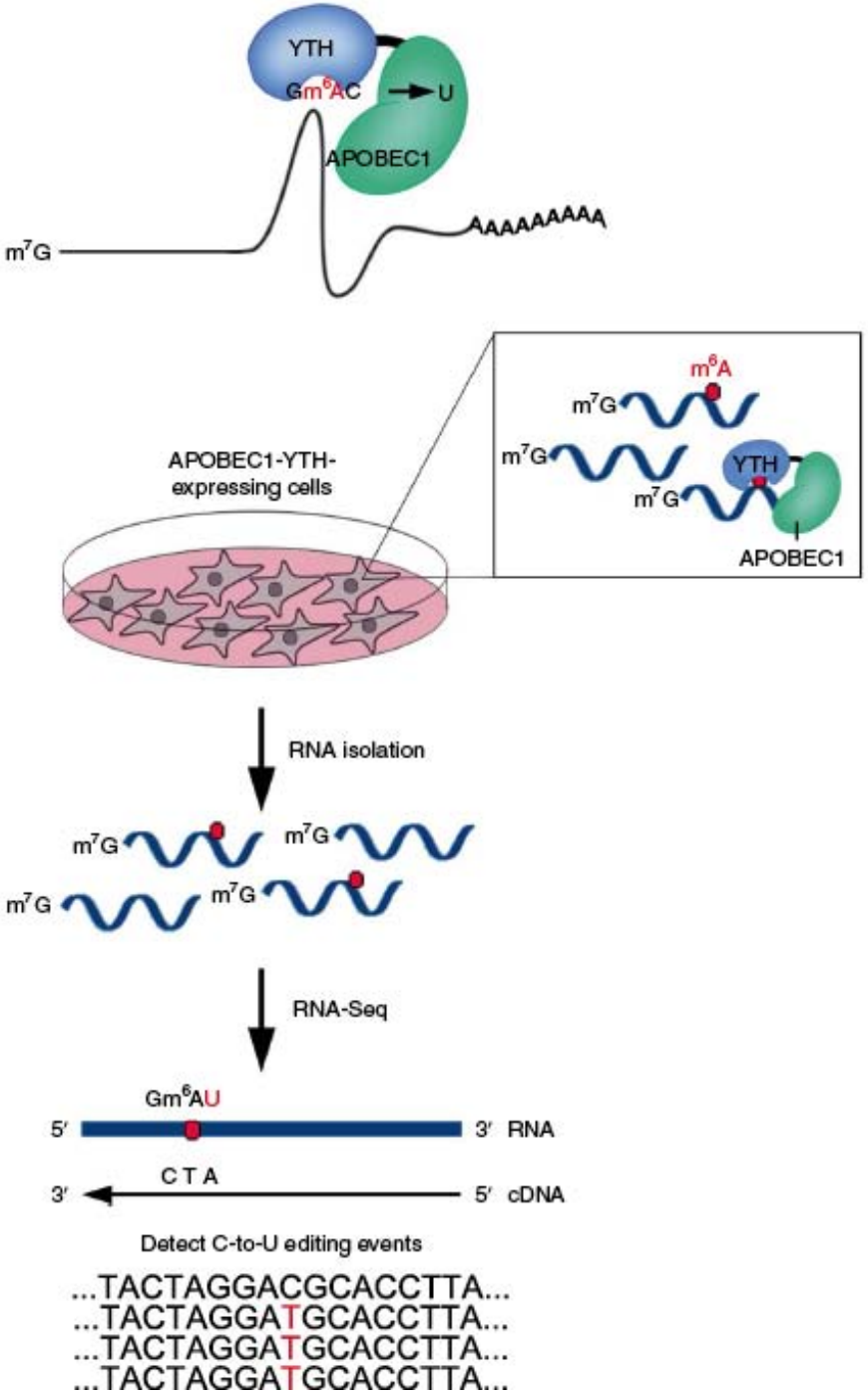
m⁶A RNA-seq



Li at al, Nat Methods, 2017

antibody-free m6A-seq DART-seq

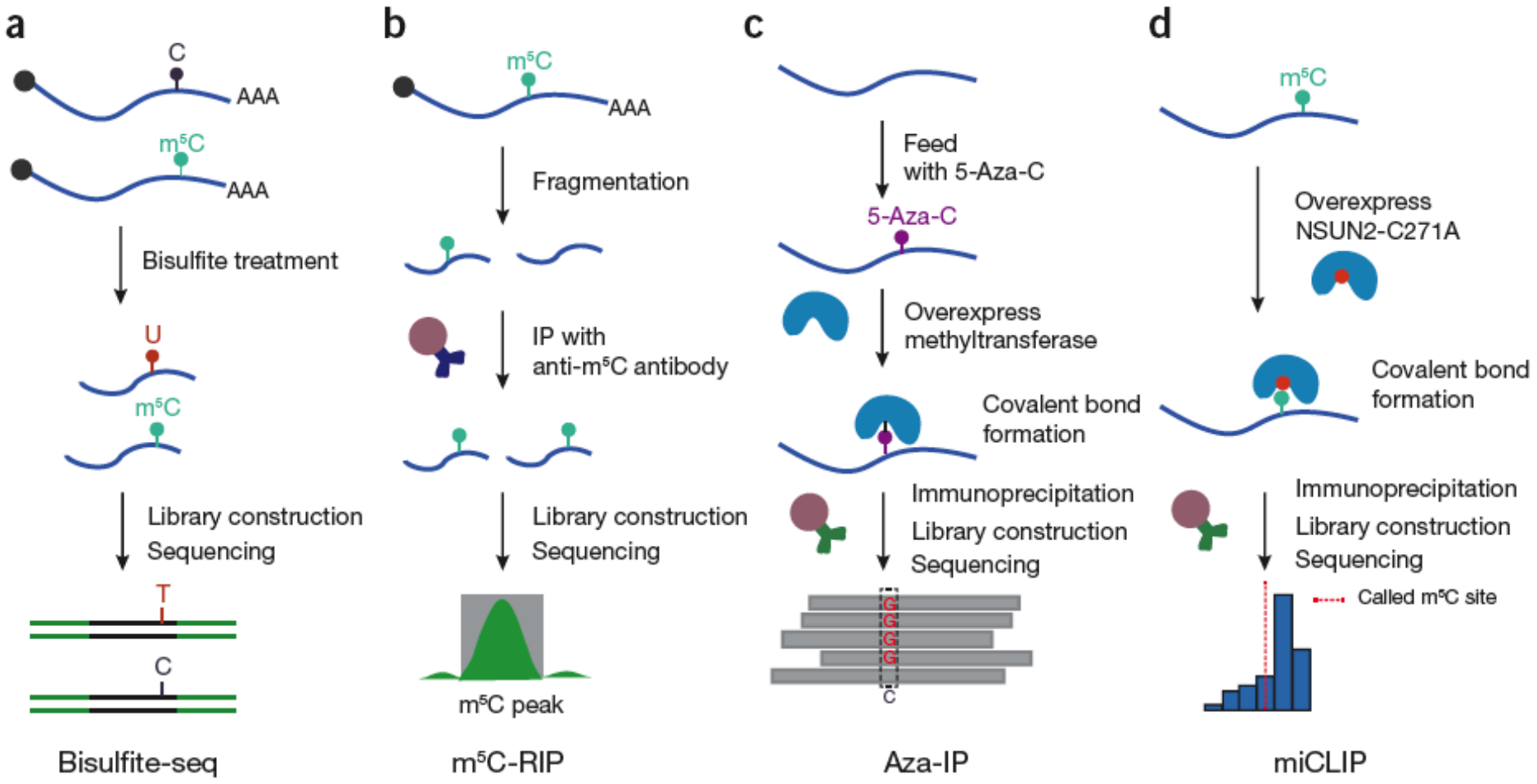
deamination adjacent to RNA modification targets



- **Cytidine deaminase APOBEC1 fused to m^{6}A-binding YTH domain**
- **APOBEC1-YTH induces C-to-U deamination at sites adjacent to m6A**
- **detected using RNA-seq**

Meyer, Nature Methods 2019

m^{5}C RNA-seq

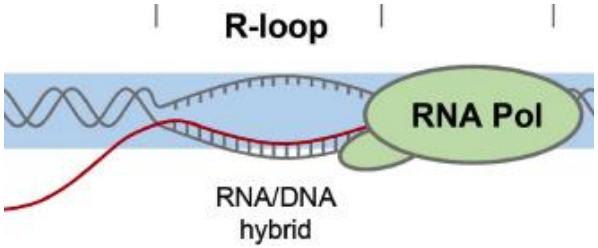


Li at al, Nat Methods, 2017

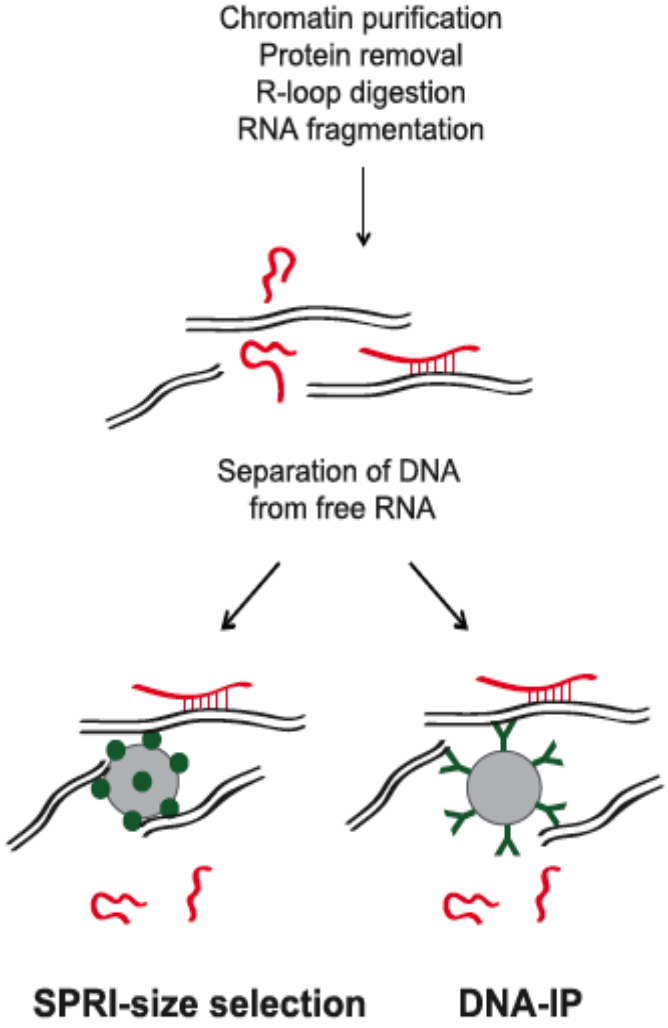
Detection of RNA-DNA hybrids

R-loop

IP with specific RNA/DNA S9.6 Ab followed by RNAseq



RNA/DNA tripplex

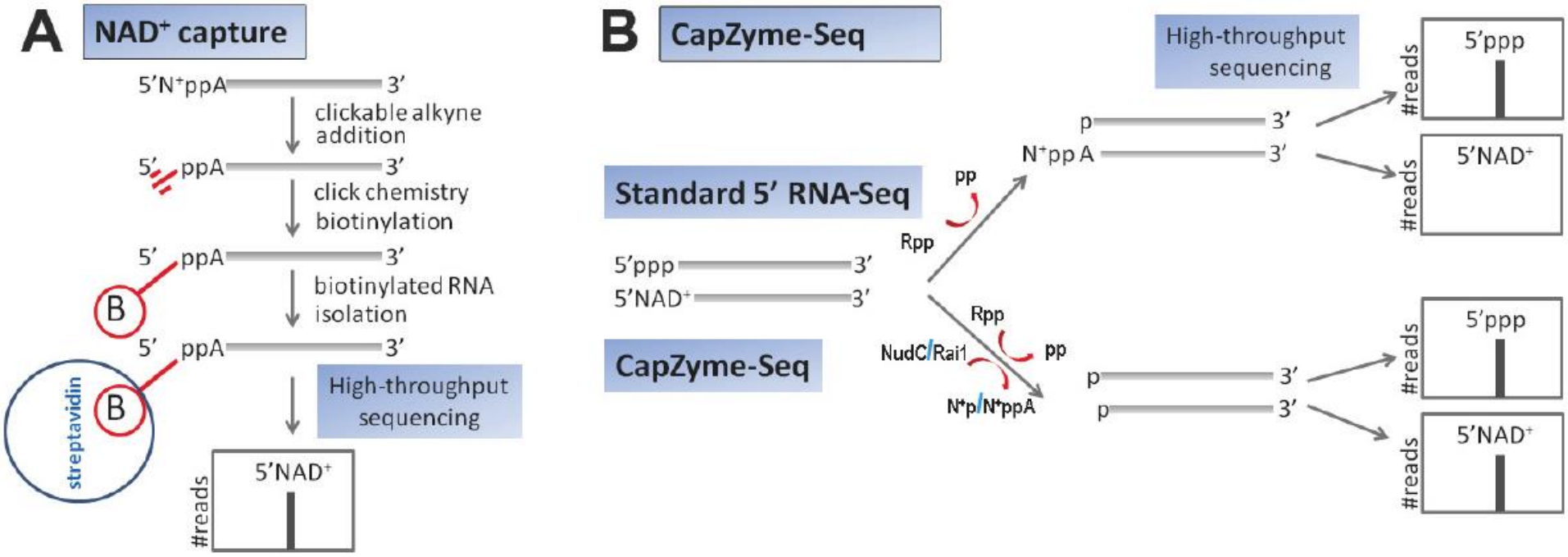


Enrichment of DNA-associated RNA

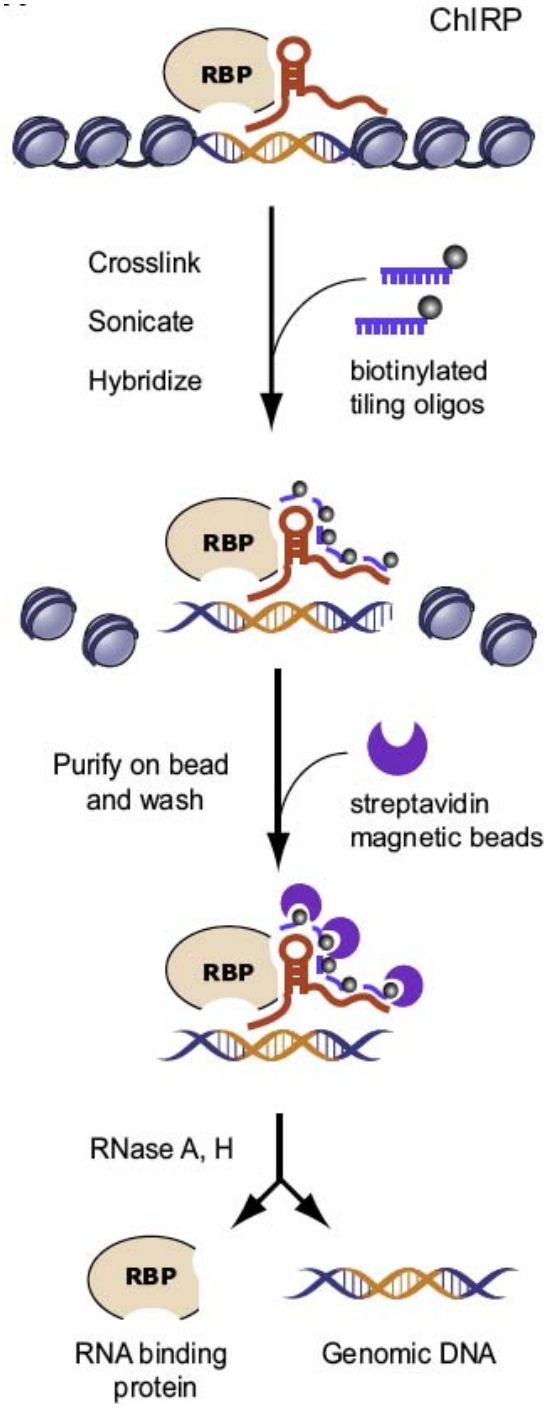
- SPRI (Solid Phase Reversible Immobilization) - based paramagnetic bead size selection
- DNA-IP using anti-DNA Ab

Cetin at al, RNA, 2019

Identification of NAD+ capped RNAs



Chromatin Isolation by RNA Purification

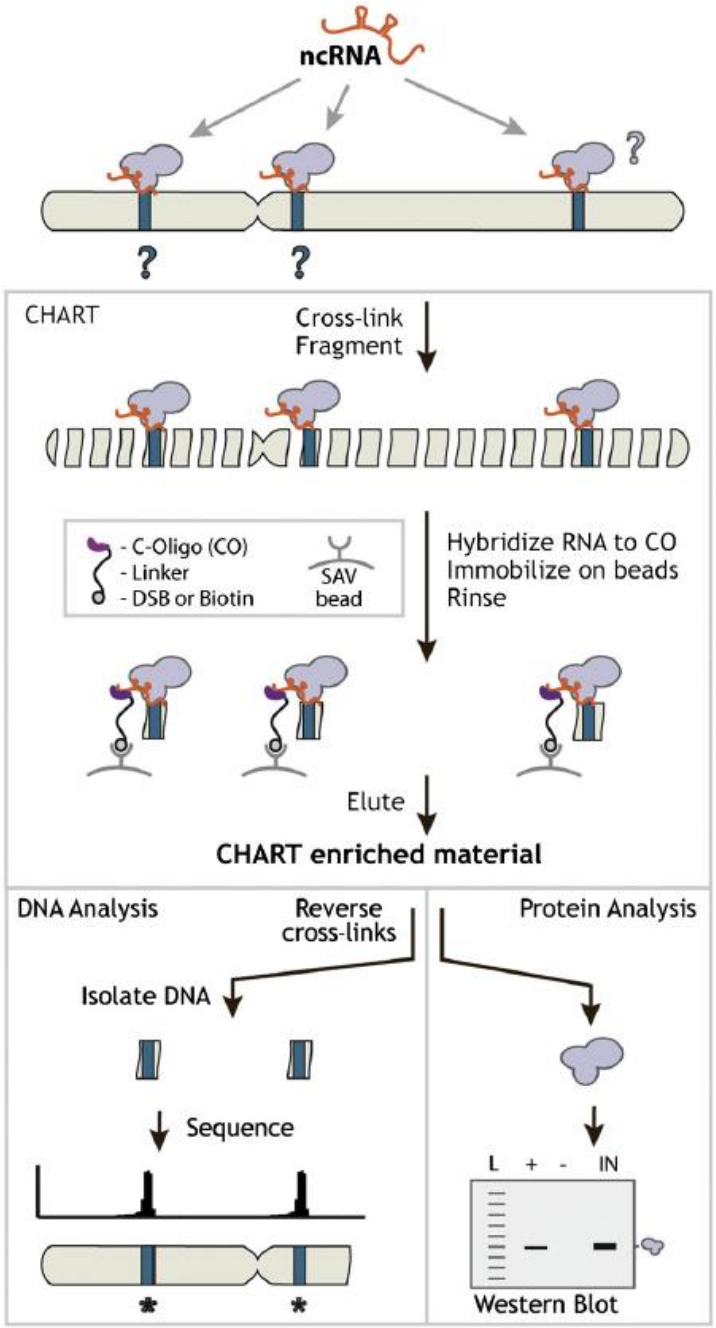


lncRNA
proteins
DNA

ChIRP

CHART

Capture Hybridization Analysis of RNA Targets

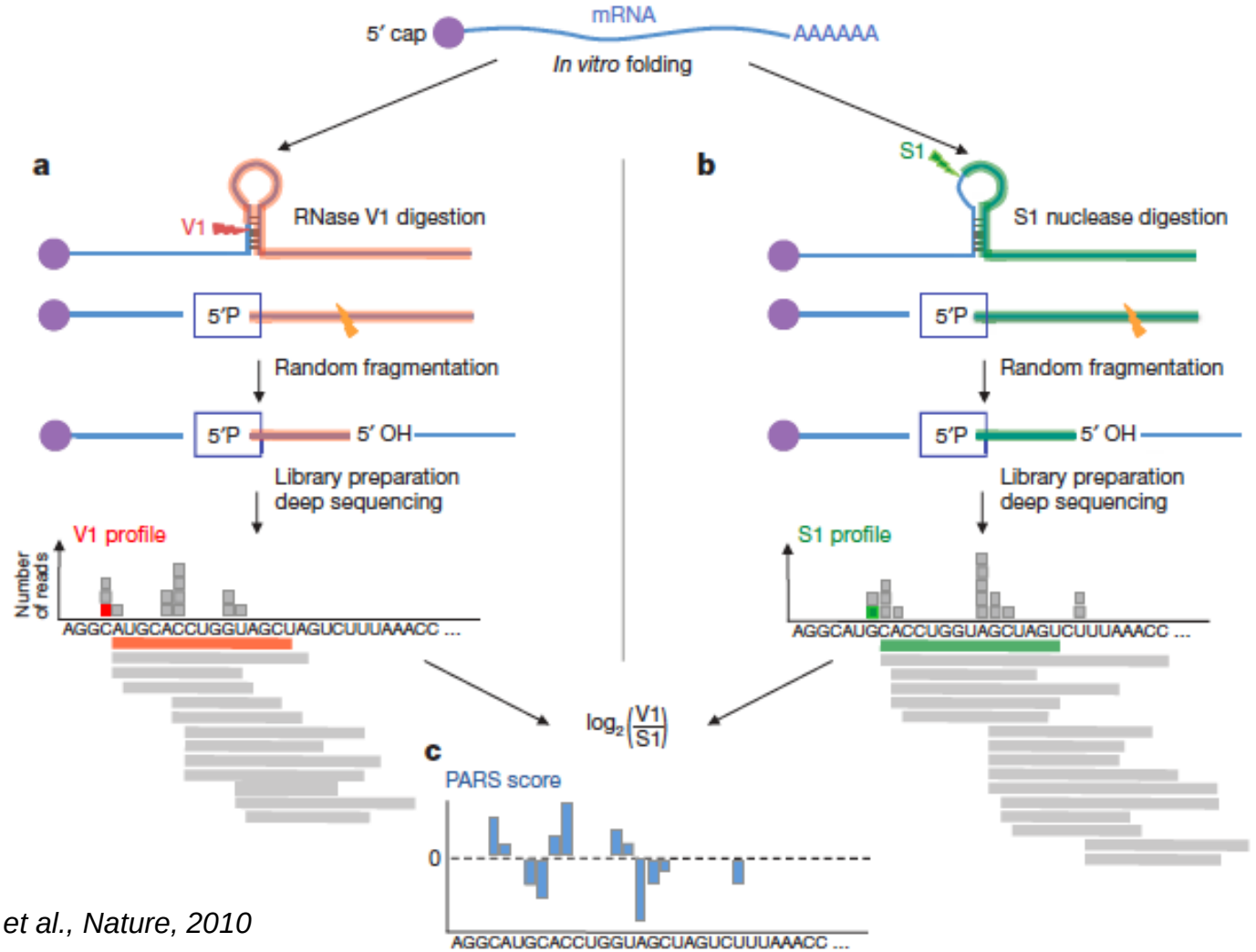


Chu et al., Mol. Cell, 2011; Simon et al., PNAS '11

PARS: Parallel Analysis of RNA Structure

measuring RNA structural properties by deep sequencing

- PARS confirmed for known RNA structures
- used to establish structures of > 3000 yeast transcripts
- unexpected conclusion: coding mRNA regions are more structured than UTRs!



Kertesz et al., Nature, 2010

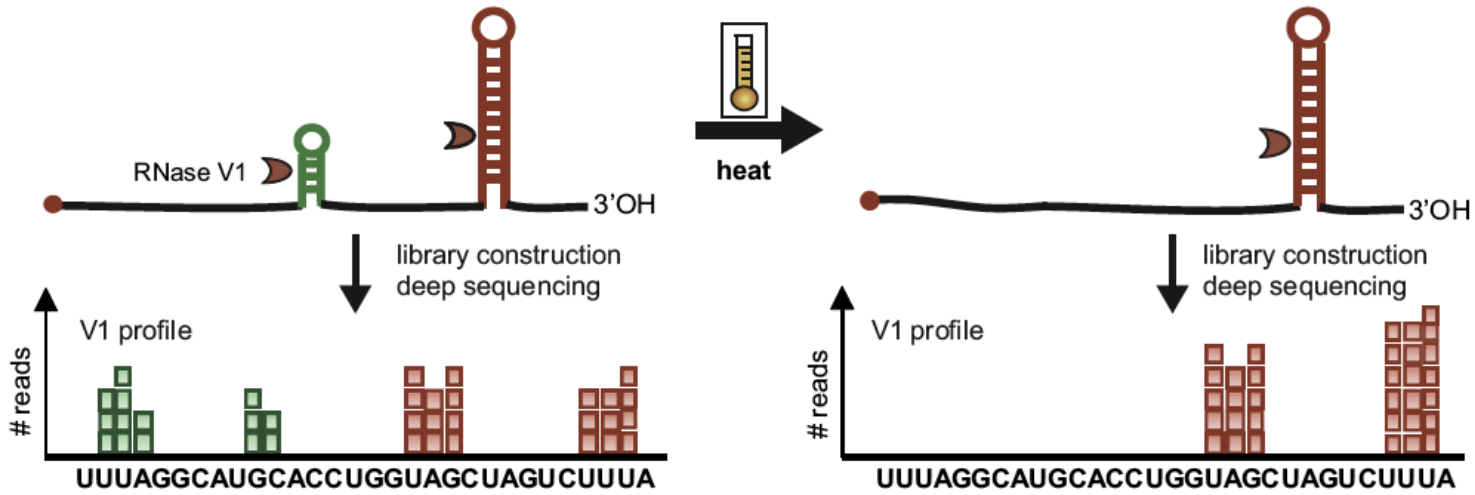
PARS: Parallel Analysis of RNA Structure

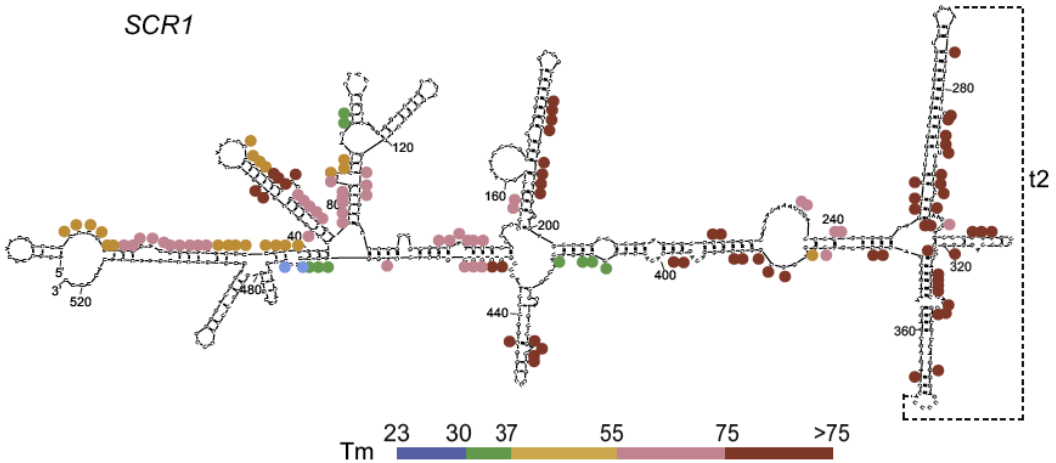
Molecular Cell
Article

Genome-wide Measurement of RNA Folding Energies

Molecular Cell *48*, 169–181, October 26, 2012

Yue Wan,[1] Kun Qu,[1,8] Zhengqing Ouyang,[1,2,8] Michael Kertesz,[3] Jun Li,[4] Robert Tibshirani,[4] Debora L. Makino,[5] Robert C. Nutter,[6] Eran Segal,[7,*] and Howard Y. Chang[1,*]





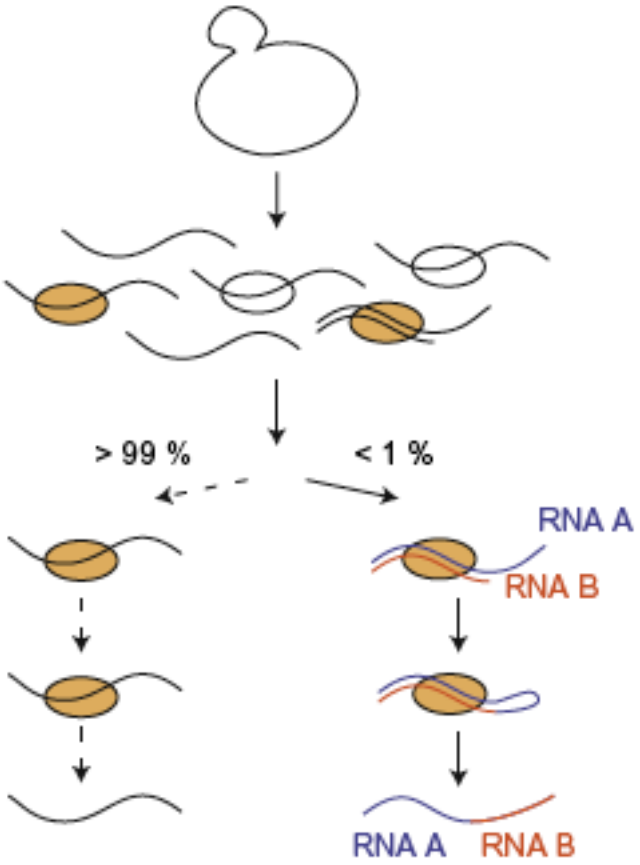
Regulatory Impact of RNA Secondary Structure across the *Arabidopsis* Transcriptome [W][OA]

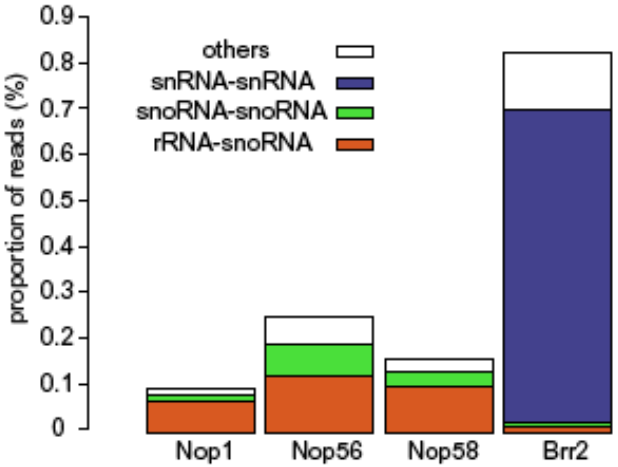
The Plant Cell 2012

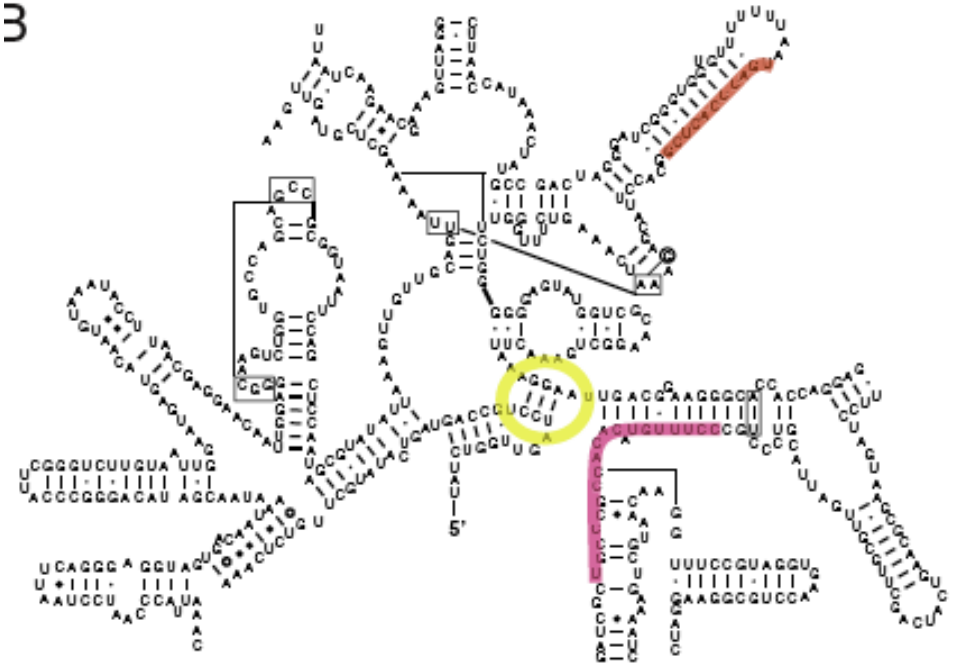
Fan Li,[a,b,c,1] Qi Zheng,[a,b,1] Lee E. Vandivier,[a,b,d] Matthew R. Willmann,[a,b] Ying Chen,[a,b,c] and Brian D. Gregory[a,b,c,d,2]

CLASH (intra- and intermolecular RNA-RNA interactions)

Crosslinking Ligation and Sequencing of Hybrids





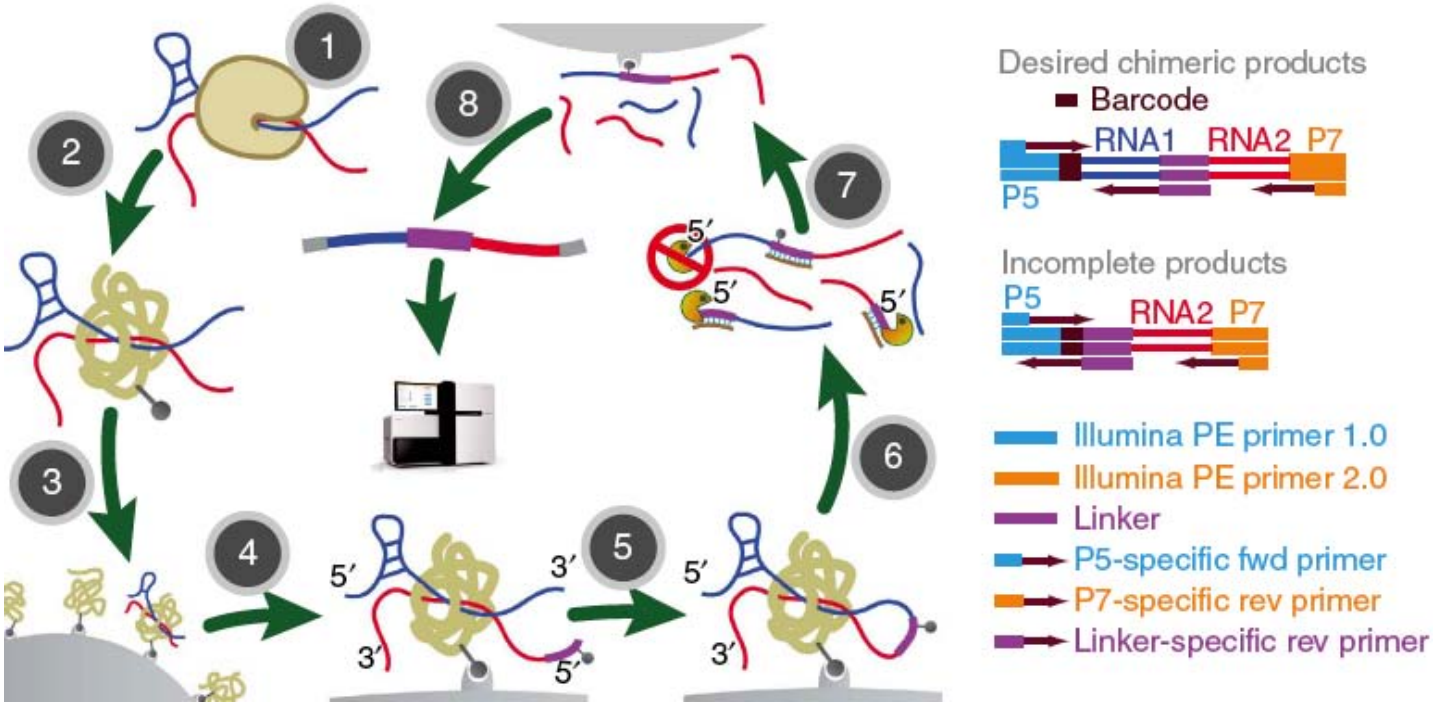


U3- 18S rRNA interactions

Kudla et al., PNAS, 2011

MARIO (intra- and intermolecular RNA-RNA interactions)

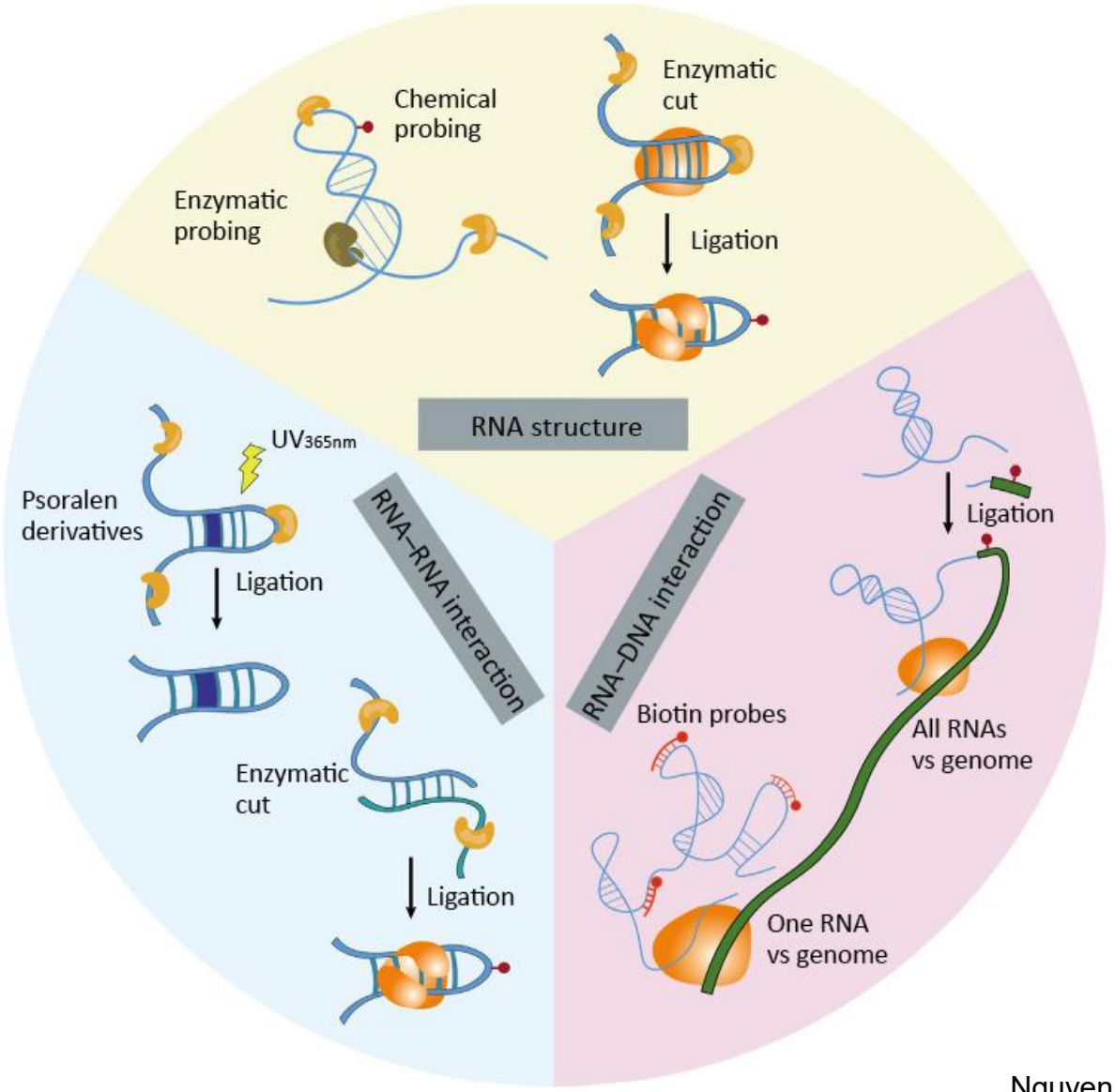
Mapping RNA interactome in vivo



(1) cross-linking RNAs to proteins
(2) RNA fragmentation, protein denaturing and biotinylation
(3) immobilization of RNA-binding proteins at low density
(4) ligation of a biotinylated RNA linker
(5) proximity ligation under a dilute condition
(6) RNA purification and RT
(7) biotin pull-down
(8) construction of sequencing library

Nguyen et al., NatComm, 2016

RNA-seq-based methods for mapping RNA structures, RNA–RNA and RNA–DNA interactions



Nguyen et al, *TiG, 2018*

RNA structure *in vivo*: SHAPE, PARIS/SPLASH/LIGR

Chemical and enzymatical-based structure probing

SHAPE: Selective 2′- Hydroxyl Acylation and Primer Extension

SHAPE-seq: SHAPE followed by RNA-seq

PARIS: Psoralen Analysis of RNA Interactions and Structures

SPLASH: Sequencing of Psoralen crosslinked, Ligated, and Selected Hybrids

LIGR-seq: LIGation of interacting RNA followed by high-throughput Sequencing

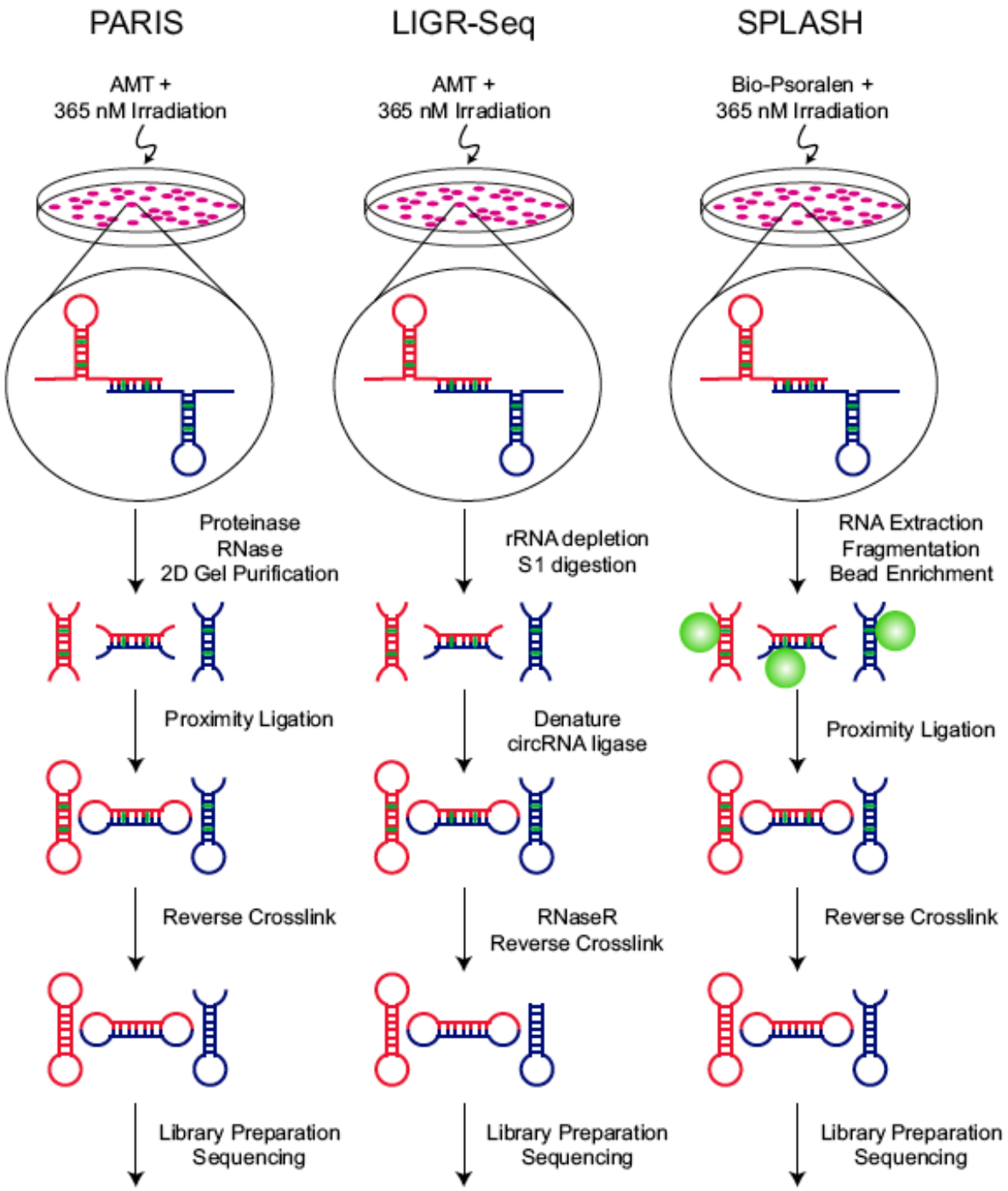
SHAPE chemicals: DMS, dimethyl sulfate; 1M7, 1-methyl-7-nitroisatoic anhydride

SHAPE enzymes: P1 nuclease, RNases V1 and S1

Table 1. Transcriptome-wide RNA Structure Probing Methods

Assay	Probing Agent	Detection	In Vitro Probing	In Vivo Probing
FragSeq	P1 nuclease	single-stranded bases	X	
PARS	RNase V1 and S1 nuclease	paired and single-stranded regions	X	
SHAPE-seq	1M7	single-stranded bases	X	
mod-seq	DMS	unpaired A & C		X
DMS-seq	DMS	unpaired A & C	X	X
Structure-seq	DMS	unpaired A & C	X	X
icSHAPE	NAI-N_3	single-stranded bases		X
SHAPE-MaP	1M7	single-stranded or unbound bases	X	X
PARIS	AMT	base-paired sequence partners		X
LIGR-seq	AMT	base-paired sequence partners		X
SPLASH	biotinylated psoralen	base-paired sequence partners		X

Graveley, Mol Cell, 2016



PARIS
SPLASH
LIGR

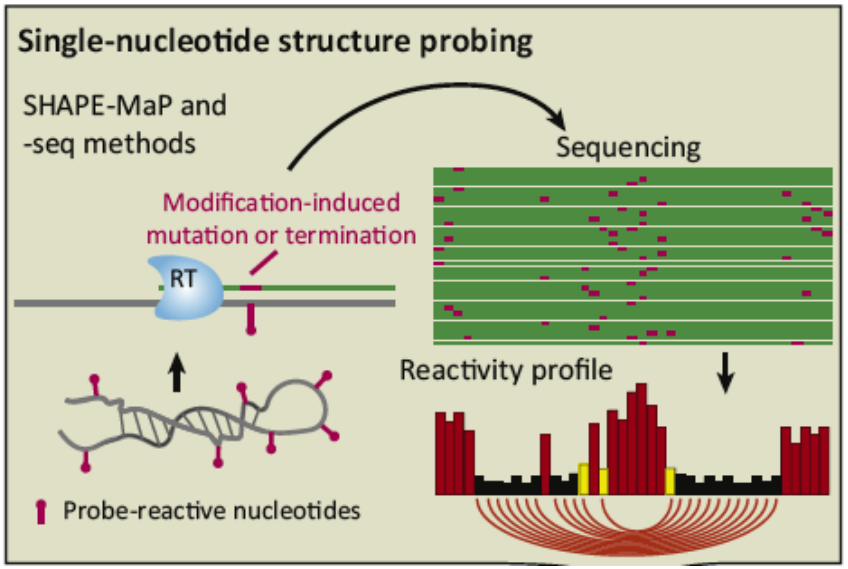
- in vivo psoralen or AMT, intercalate into RNA duplex and generate inter-strand adducts between juxtaposed pyrimidine bases upon 365 nm UV irradiation
- ssRNAse S1 limited digest
- RNA end proximity ligation (circRNA ligase)
- removal of uncrosslinked RNA (ss and structured RNAase R1)
- crossling reversal (254 nm)
- RNAseq

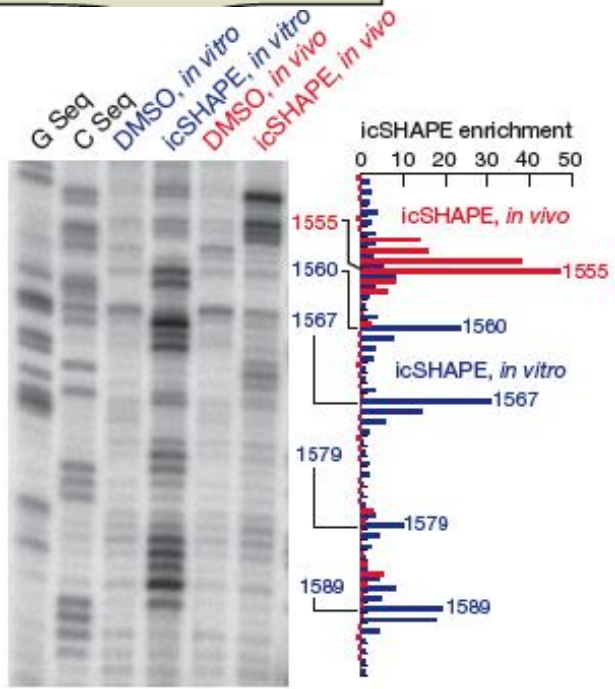
[AMT = psoralen derivative 4'-aminomethyltrioxalen] and

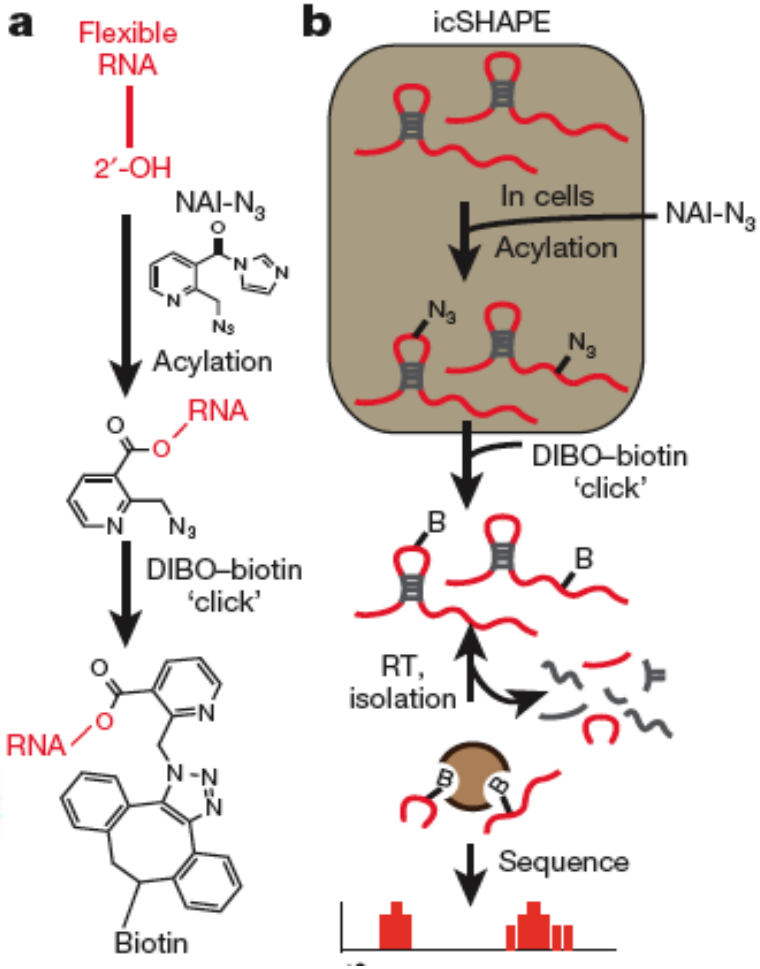
Graveley, Mol Cell, 2016

RNA structure in vivo: SHAPE, icSHAPE

icSHAPE: click selective 2'-hydroxyl acylation and profiling

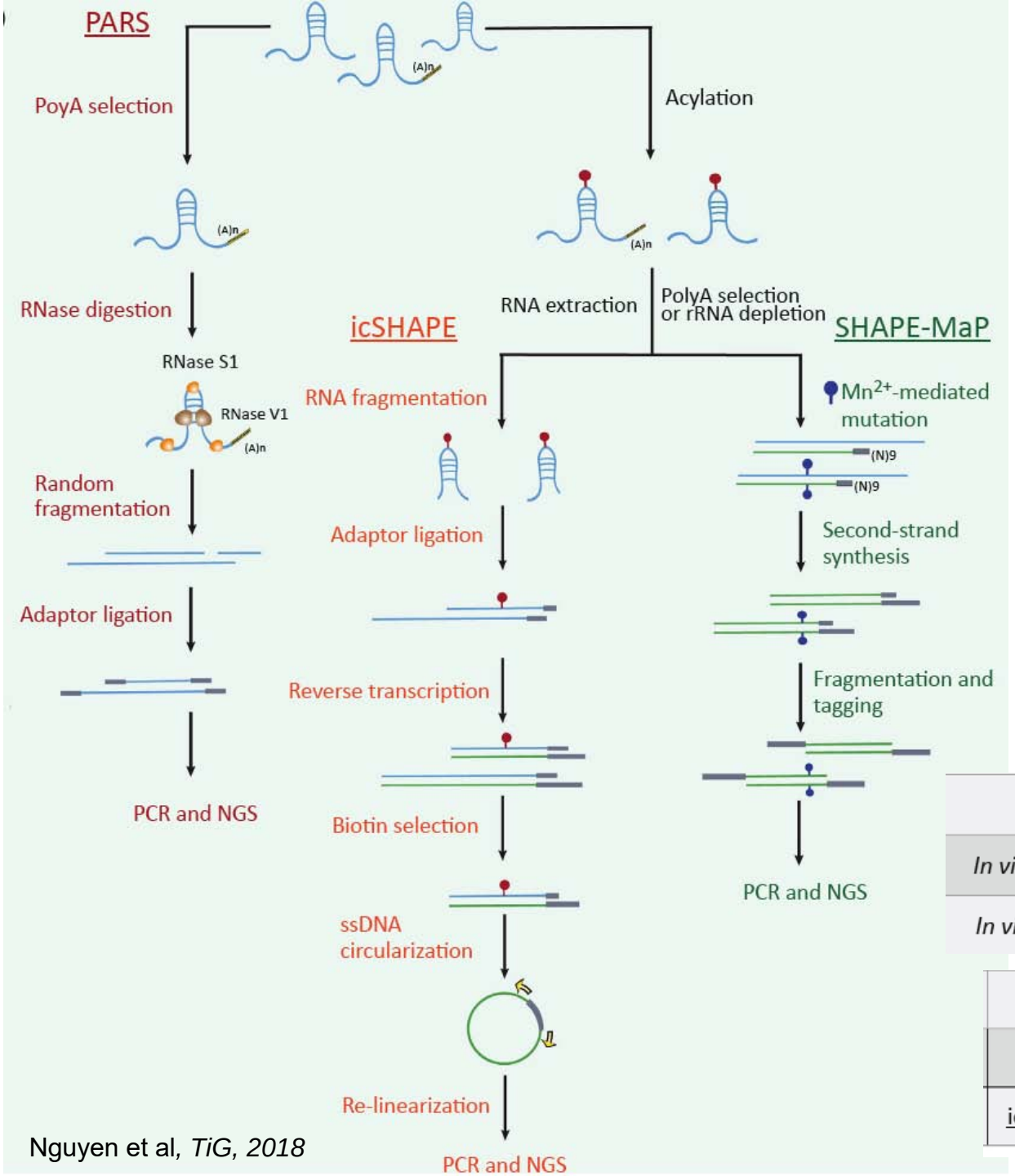






Low and Weeks, Methods 2010
Weidman et al, TiBS, 2016

Mapping RNA structures and RNA-RNA interactions



Nguyen et al, *TiG, 2018*

	Enzyme based			
In vitro	PARS	PIP-seq	PARTE	FragSeq
In vivo	MARIO			

Chemical based			
SHAPE-MaP		CIRS-seq	
icSHAPE	Structure-seq	DMS-seq	Mod-seq

RNA-seq-based mapping of RNA structures

Sequencing based for mapping RNA structures		
Method	Advantages	Limitations
Enzyme-based methods		
PIP-seq	• Reveals both protein-bound RNA regions and RNA secondary structure. • Provides strand-specific information.	• Limited resolution at small nucleotide bulges and loops.
PARS	• Increased sensitivity by sequencing both single- and double-stranded regions.	• RNA was folded *in vitro*.
PARTE	• Measures melting temperature. • Single-nucleotide resolution. • Preserves *in vivo* RNA modifications. • Can infer RNA regulatory motifs.	
FragSeq	• Simple and fast protocol. • Accompanied with modifiable software.	
Chemical-based methods		
DMS-seq	• Identifies RNA structure in native conditions. • Single-nucleotide resolution.	• Limited to the analysis of two bases (As and Cs). • RNA-binding proteins can block DMS activity.
icSHAPE	• Measures base flexibility. • Single-nucleotide resolution.	• Limited to the analysis of relatively short (~300 nt) *in vitro*-transcribed RNAs.
Structure-seq	• Single-nucleotide resolution. • Applicable to both *in vitro* and *in vivo* analyses.	• Limited to the analysis of two bases (As and Cs). • RNA-binding proteins can block DMS activity.
Mod-seq	• Can probe structures of long RNAs *in vivo*. • Single-nucleotide resolution.	• Limited to the analysis of two bases (As and Cs).
CIRS-seq	• Single-nucleotide resolution. • Can identify structural requirements for RNA-binding proteins.	
SHAPE-MaP	• Can be customized for different applications. • Applicable to analysis of long RNAs. • Can infer structural changes of single-nucleotide and other allelic polymorphisms.	• Length of the RNA must be at least ~150 nt for the randomer and native workflow, and at least ~40 nt for the small-RNA workflow.

Nguyen et al, *TiG, 2018*

RNA-seq-based mapping of RNA-RNA interactions

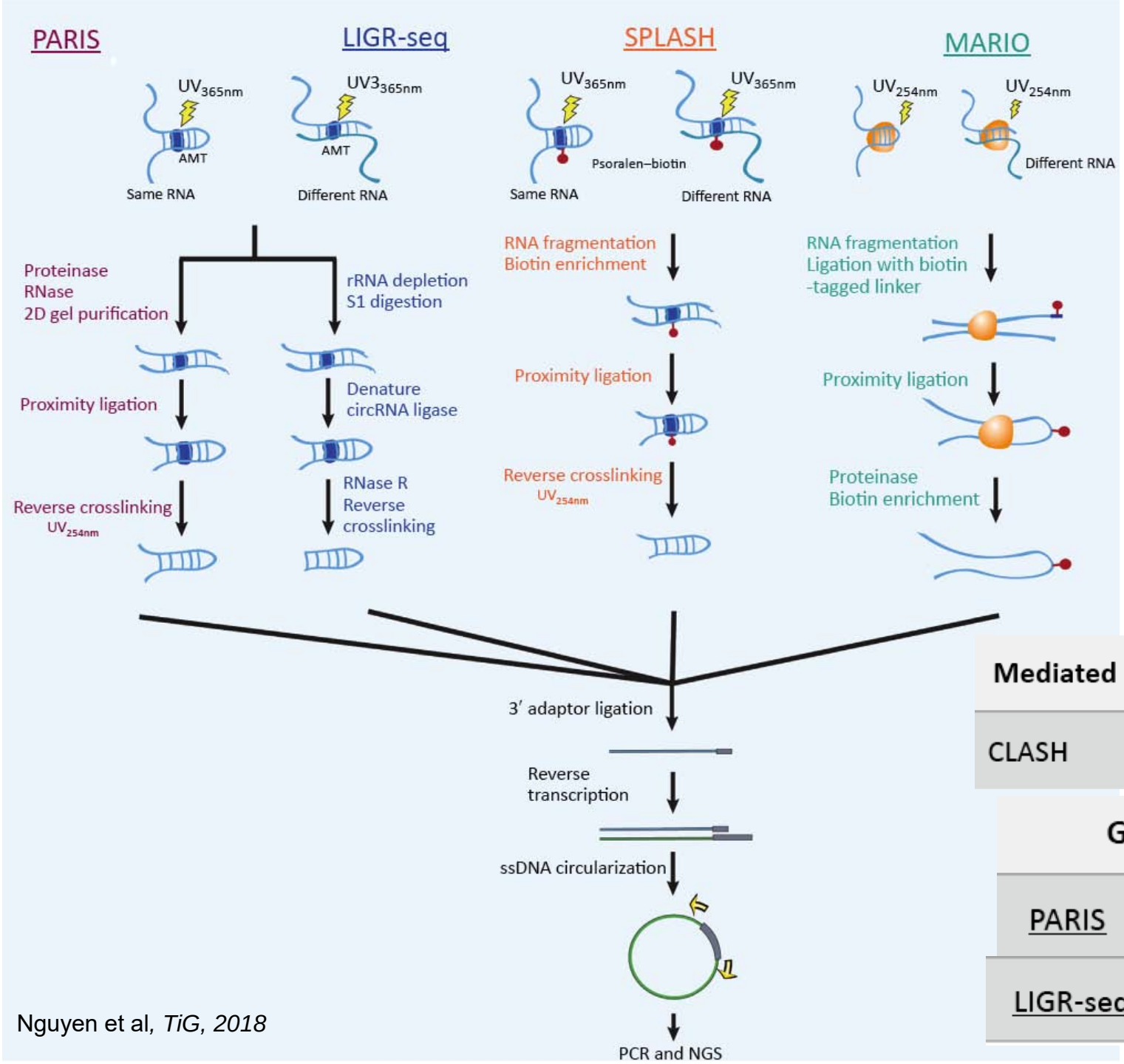
Sequencing based for mapping RNA–RNA interactions		
CLASH	• Stringent purification conditions remove nonphysiological interactions.	• Requires prior knowledge of an RNA-binding protein. • Requires a good antibody.
hiCLIP	• Incorporation of an adaptor between two RNA molecules increases ligation efficiency and improves accuracy in sequence mapping.	• Requires prior knowledge of an RNA-binding protein. • Requires a good antibody. • No *in vivo* crosslinking step may incur challenges in differentiating *bona fide* and spurious RNA attachments.
PARIS	• Many-to-many mapping.	• 4′-Aminomethyl trioxsalen (AMT) preferentially crosslinks pyrimidine bases and may introduce bias.
SPLASH	• Improves signal-to-noise ratio by leveraging biotinylated psoralen. • Many-to-many mapping.	• Psoralen preferentially crosslinks pyrimidine bases and may introduce bias.
LIGR-seq	• Many-to-many mapping.	• AMT preferentially crosslinks pyrimidine bases and may introduce bias.
MARIO	• Many-to-many mapping. • Incorporation of an adaptor between two RNA molecules increases ligation efficiency and improves accuracy in sequence mapping. • Reports both between- and within-molecule interactions.	• Loses RNA duplexes that are not associated with any proteins.
	• Captures proximal regions of an RNA molecule in 3D. • Reveals single-stranded regions of each RNA.	

Nguyen et al, *TiG, 2018*

RNA-seq-based mapping of RNA-DNA interactions

Sequencing based for mapping RNA–DNA interactions		
Method	**Advantages**	**Limitations**
	• Captures proximal regions of an RNA molecule in 3D. • Reveals single-stranded regions of each RNA.	
Sequencing based for mapping RNA–DNA interactions		
ChIRP	• Tilling the entire transcript with antisense DNA.	• Limited to analyzing RNA at a time.
CHART	• Tilling the RNase H accessible region by antisense DNA.	• Limited to analyzing RNA at a time.
RAP	• Tilling the entire transcript with complimentary RNA.	• Limited to analyzing RNA at a time. • Limited to analysis of long RNA.
MARGI	• Many-to-many mapping. • Captures interaction at native conditions.	• Require a large number (10^7) of cells.
ChAR-seq	• Many-to-many mapping. • Proximity ligation is performed in nuclei, which reduces nonspecific interactions.	• Only sequencing reads that cover the entire bridge sequence are informative, reducing the number of informative reads.
GRID-seq	• Many-to-many mapping. • Proximity ligation is performed in nuclei, which reduces nonspecific interactions.	• The informative sequence lengths on the RNA side and the DNA side are both limited to ~20 bases, resulting in challenges in unambiguous sequence mapping.

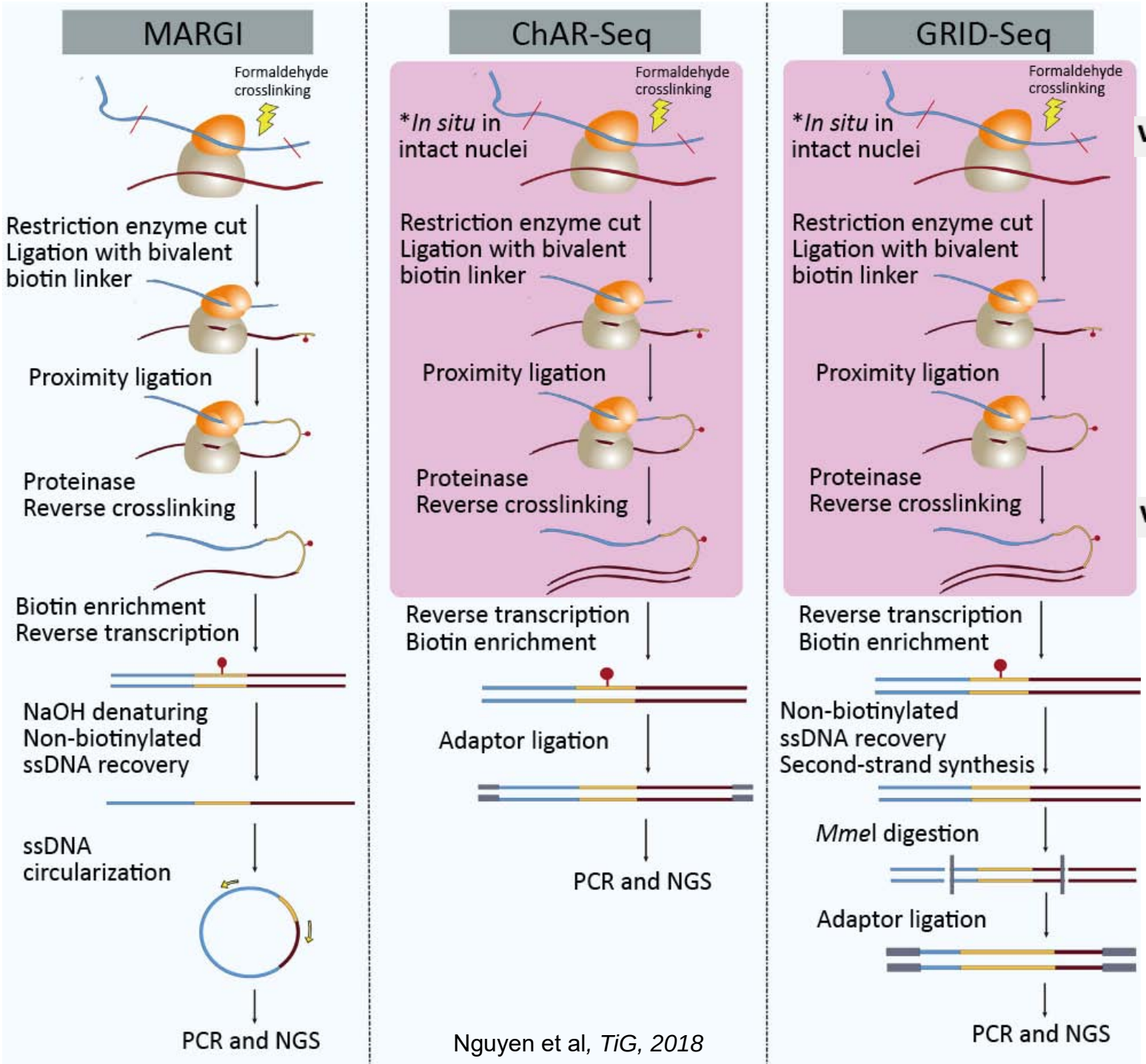
Nguyen et al, *TiG, 2018*



Mediated by a specific protein	
CLASH	hiCLIP

Genome-wide	
PARIS	SPLASH
LIGR-seq	MARIO

Nguyen et al, *TiG, 2018*



Nguyen et al, *TiG, 2018*

One RNA versus the genome

ChIRP-seq

CHART-seq

RAP-seq

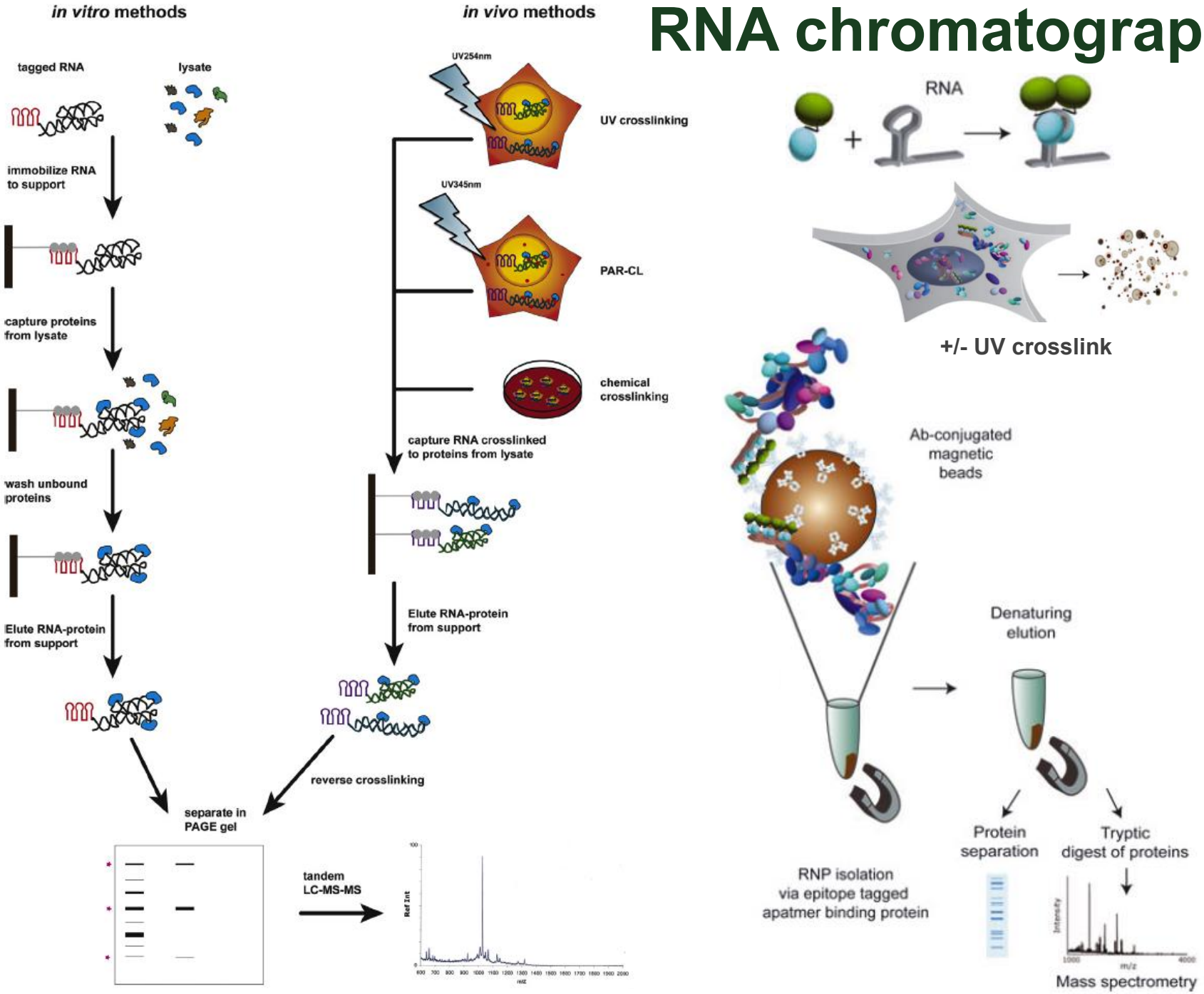
All RNAs versus the genome

MARGI

ChAR-seq

GRID-seq

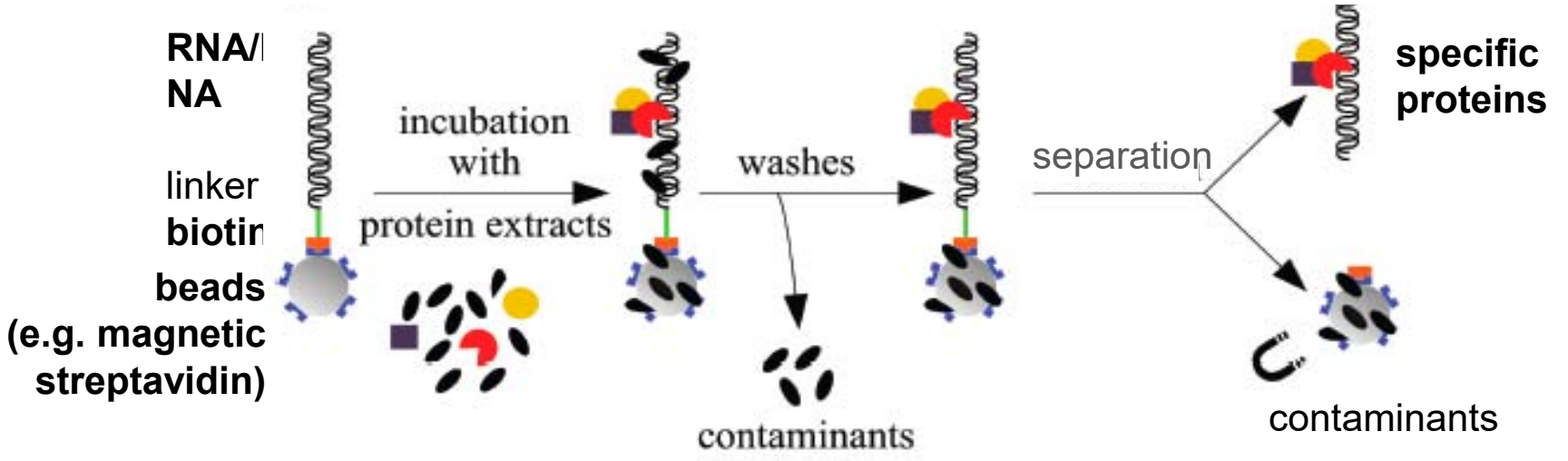
RNA chromatography



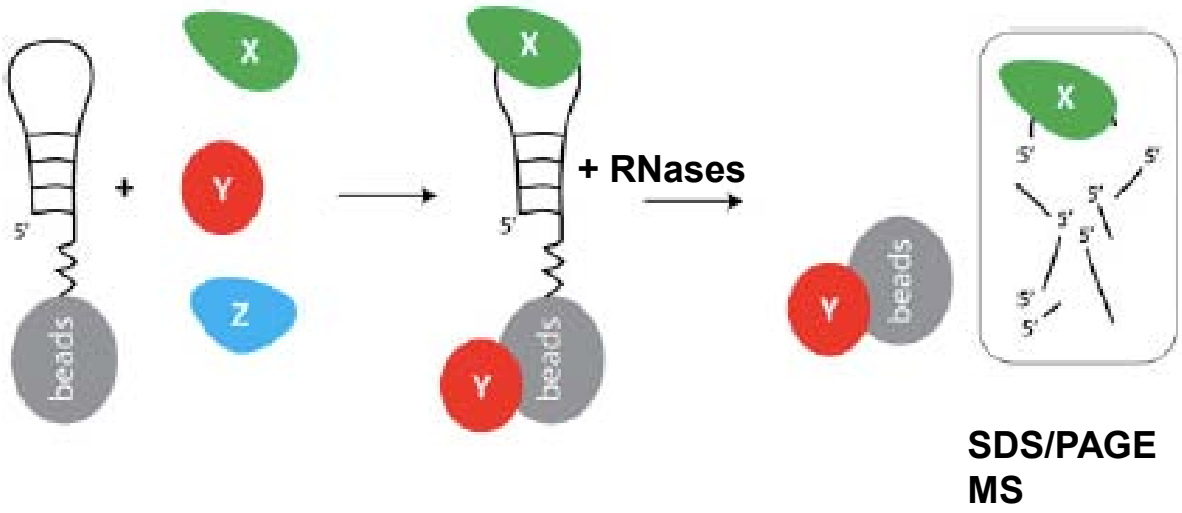
Faoro and Ataide, FEBS Lett, 2014

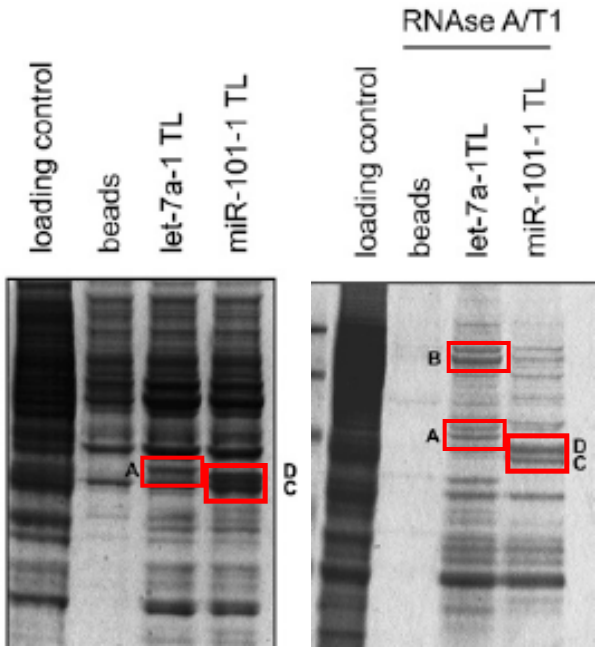
Oeffinger, Proteomics, 2012

RNA CHROMATOGRAPHY *in vitro*



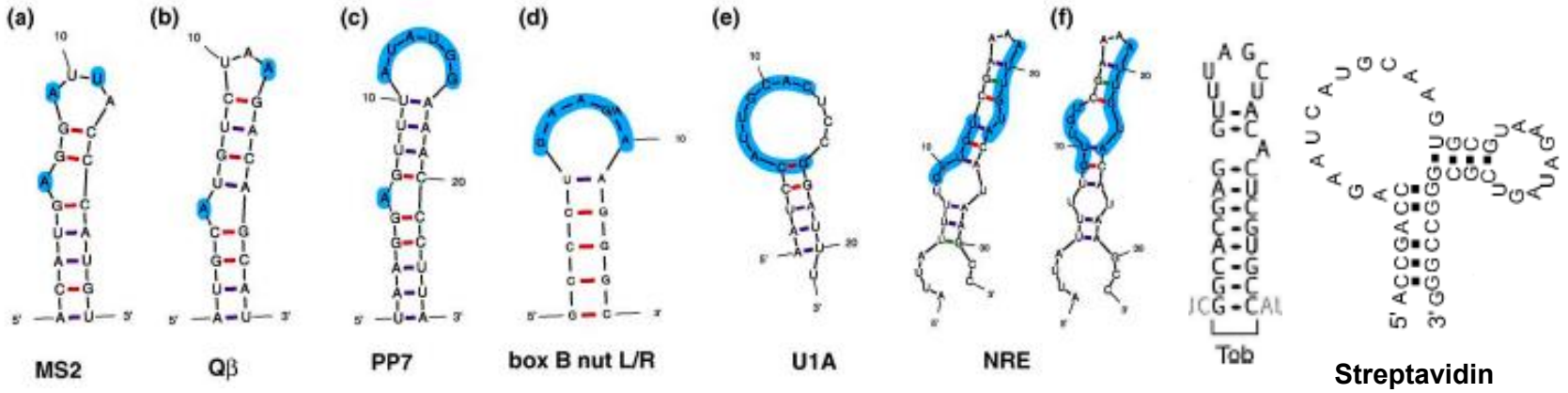
RNase-assisted RNA chromatography



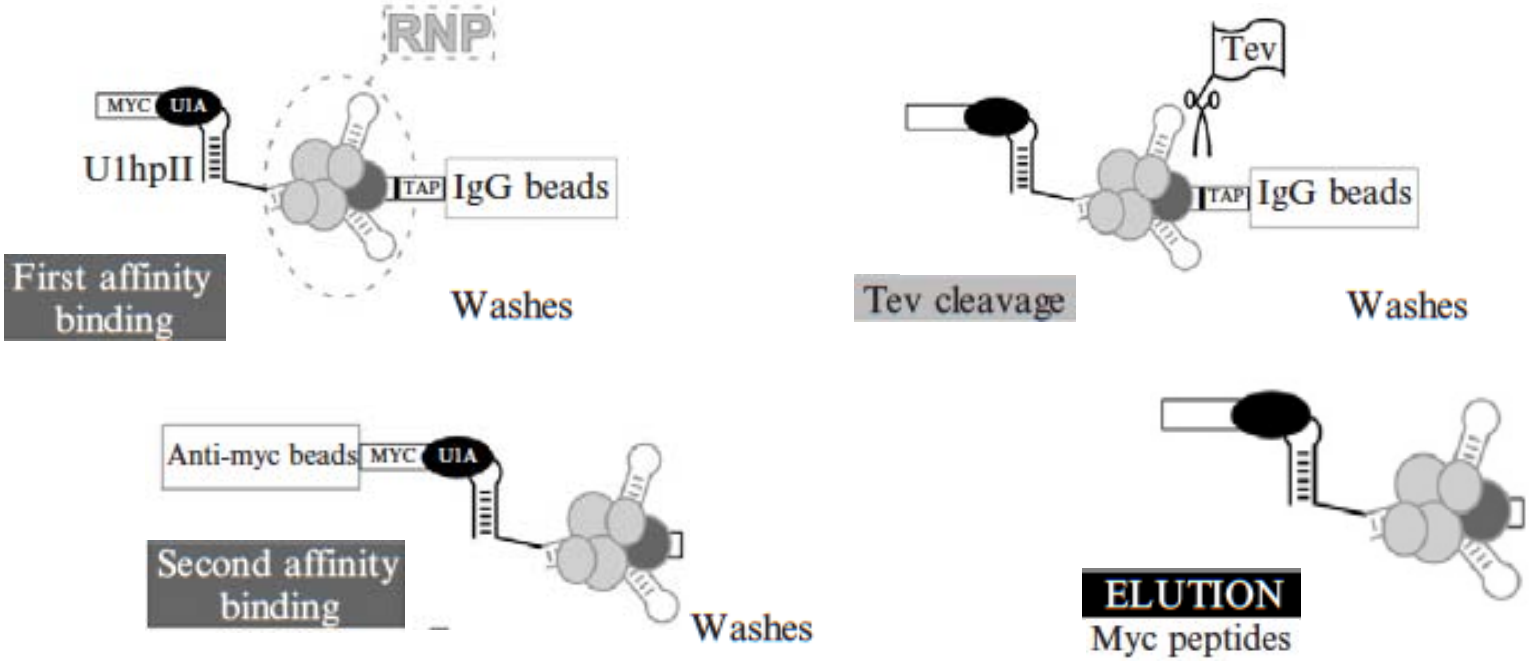


Hegarat et al., NAR, 2010; Michlewski and Caceres, RNA, 2010

RNA CHROMATOGRAPHY *in vivo*

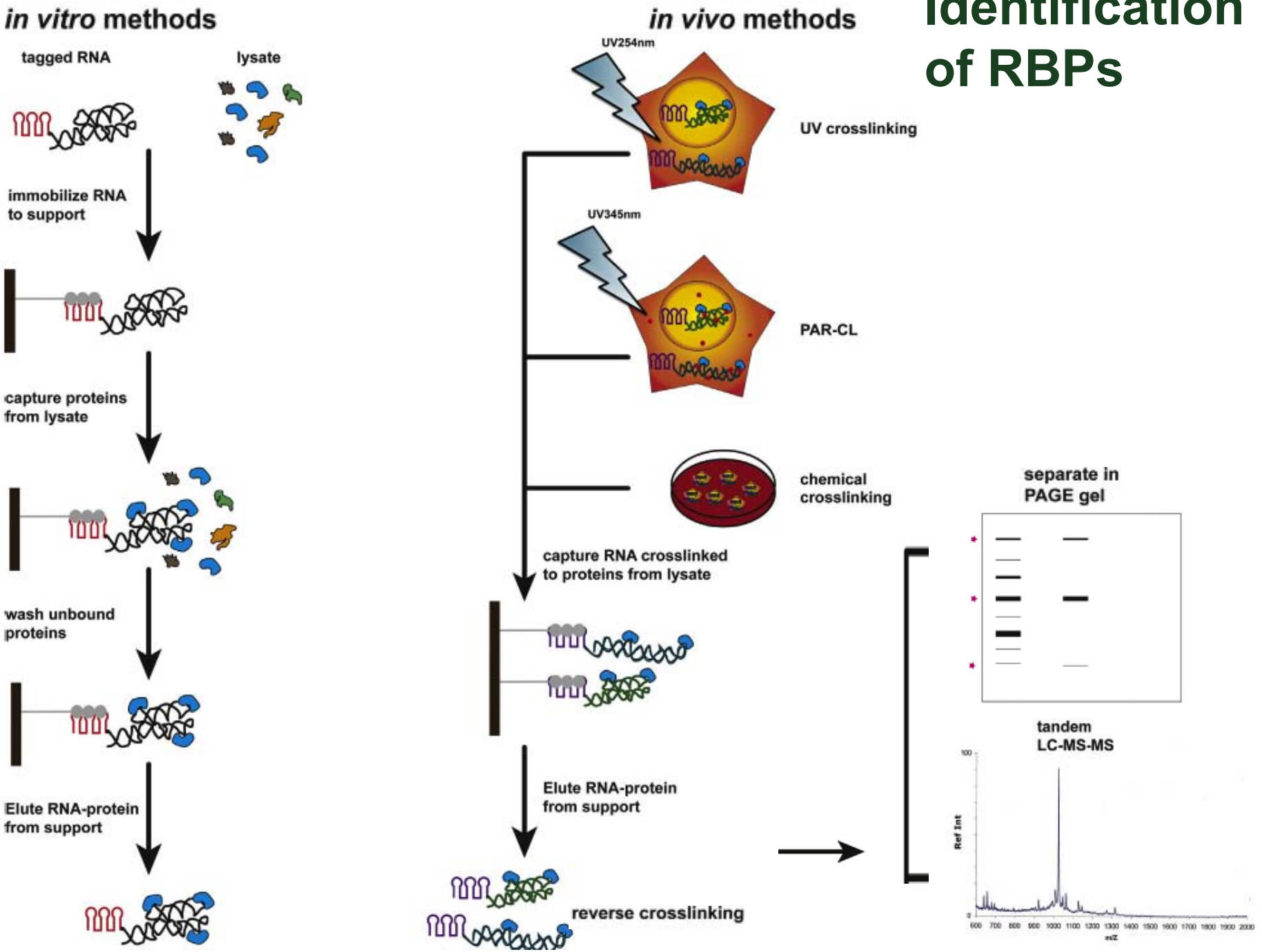


RNP purification from cells expressing RNA with affinity tags



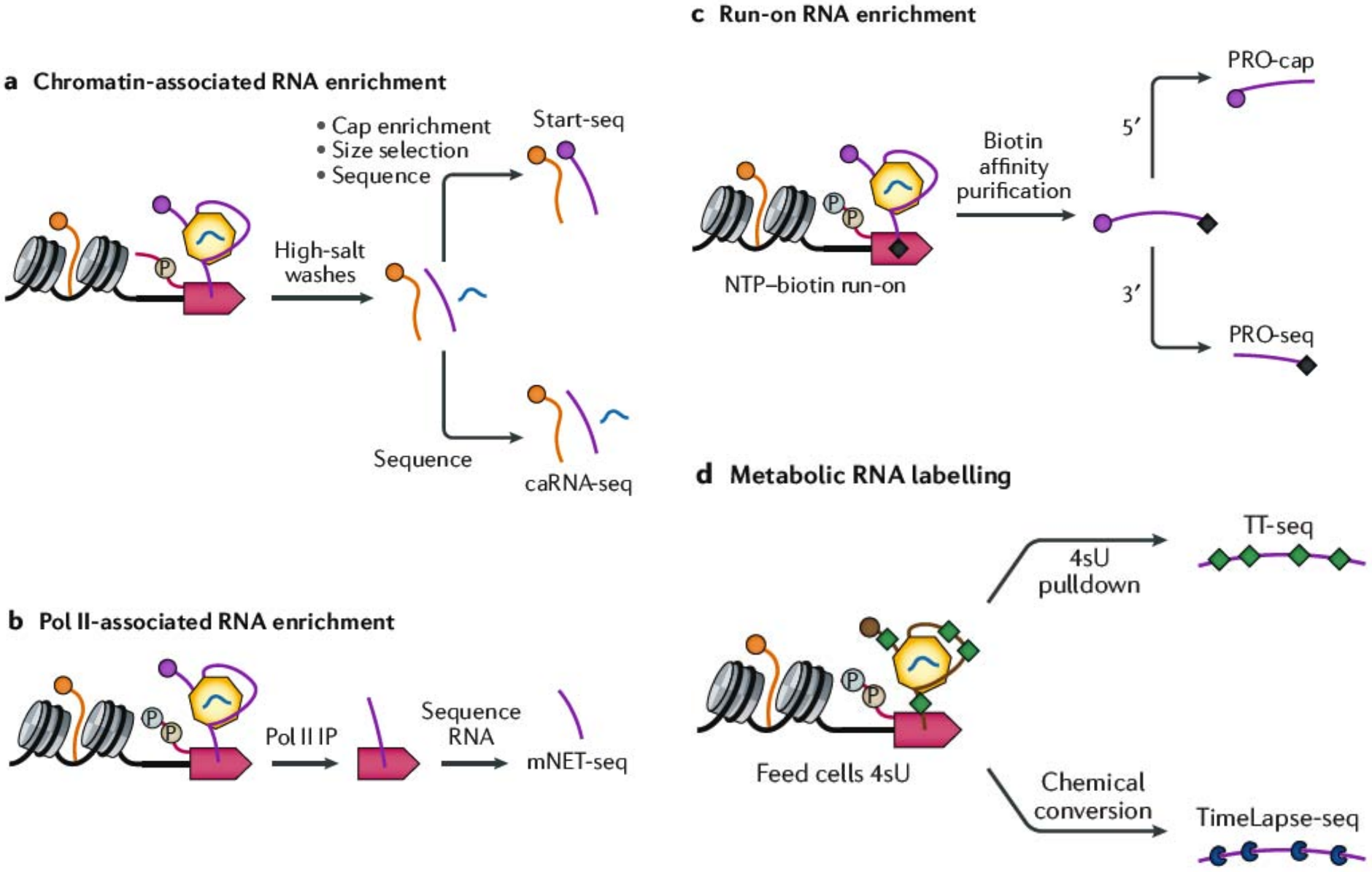
Higg and Collins, RNA, 2007; Srisawat and Engelke, Methods, 2002; Bachler et al., RNA, 1999; Weil et al., TiCB, 2010; Piekna-Przybylska et al., Meth Enzymol, 2007

Identification of RBPs



Faoro and Ataide, FEBS Lett, 2014

Nascent RNA analyses



Wissink et al, Nat Rev Genet, 2019

Nascent RNA methods

caRNA- seq chromatin-associated RNAseq
CoPRO coordinated precision run-on and sequencing
FISH fluorescence in situ hybridization
mNET-seq mammalian native elongating transcript seq
NET-seq native elongating transcript seq
PRO-cap precision run- on with cap selection
PRO-seq precision run- on seq
SL AM-seq thiol (SH)-linked alkylation for the metabolic sequencing of RNA
SMIT-seq single-molecule intron tracking seq
TT- seq transient transcriptome seq

Method	Advantages	Considerations
caRNA-seq	• Can be used to isolate all chromatin-associated RNA species • Can be combined with methods that assay co-transcriptional processes, including RNA methylation and editing	Also sequences non-nascent RNAs that stably associate with chromatin
Start-seq	• Simultaneously identifies initiation and pausing sites • Allows de novo calling of putative enhancers	Does not report transcription beyond the first ~100 nucleotides
Yeast NET-seq	• Is Pol II specific (antibody enrichment) • Identifies Pol II positions at nucleotide resolution genome-wide	Is limited to cells with epitope-tagged Pol II
mNET-seq	• Is Pol II specific (antibody enrichment) • Identifies Pol II positions at nucleotide resolution genome-wide • Can isolate Pol II with different post-translational modifications	• Includes RNAs that are stably associated with Pol II • Does not currently include RNA <30 nucleotides in length • Has detected eRNA transcription from previously called enhancers
PRO-cap	• Identifies transcription initiation sites • Allows de novo calling of putative enhancers	Does not report transcription beyond the first ~100 nucleotides
PRO-seq	• Captures RNAs from transcriptionally competent polymerases • Identifies positions of active transcription at nucleotide resolution genome-wide • Allows de novo calling of putative enhancers	• Does not measure polymerase backtracking • Also captures RNAs being transcribed from Pol I and Pol III
CoPRO	• Simultaneously identifies initiation and pausing sites • Measures RNA capping status	Does not measure transcription beyond promoter-proximal pause site
SMIT-seq	Measures splicing status during transcription	Limited to species with short introns
TT-seq	• Captures RNAs from actively transcribing polymerases • Can be used to determine RNA stability • Identifies transcription termination sites	• Does not detect Pol II pausing • Has detected eRNA transcription from previously called enhancers
SLAM-seq and TimeLapse-seq	• Captures RNAs from actively transcribing polymerases • Can be used to determine RNA stability	• Requires deep sequencing to measure chemical conversion rate • Long labelling times do not capture newly synthesized RNA
Intron sequential FISH	• Detects transcription of thousands of genes in single cells • Contains positional information of transcribed genes in the 3D space of the nucleus	• Does not report chromosomal positions of active Pol II complexes • Does not distinguish different steps of transcription • Requires a library of intron-targeting probes and series of hybridizations

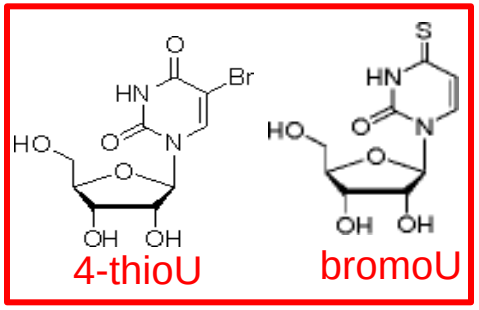
Wissink et al, Nat Rev Genet, 2019

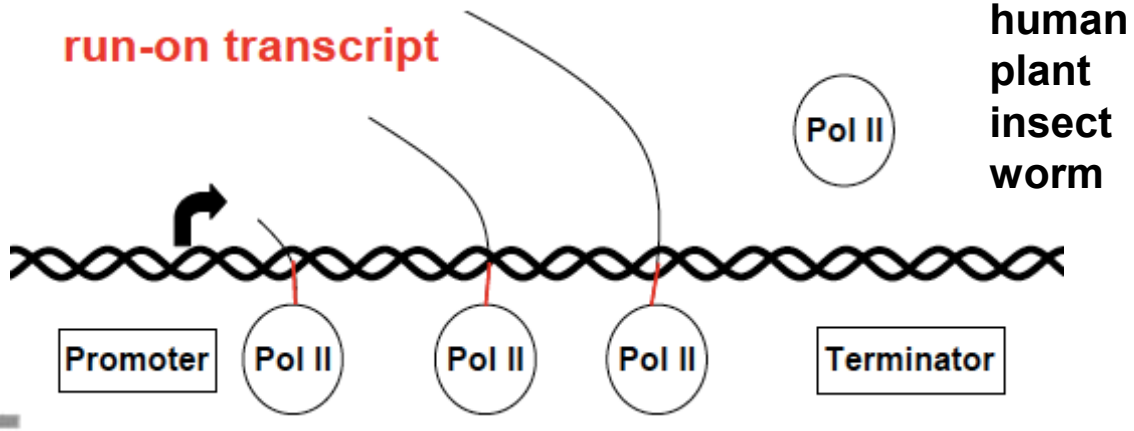
Nascent RNA methods

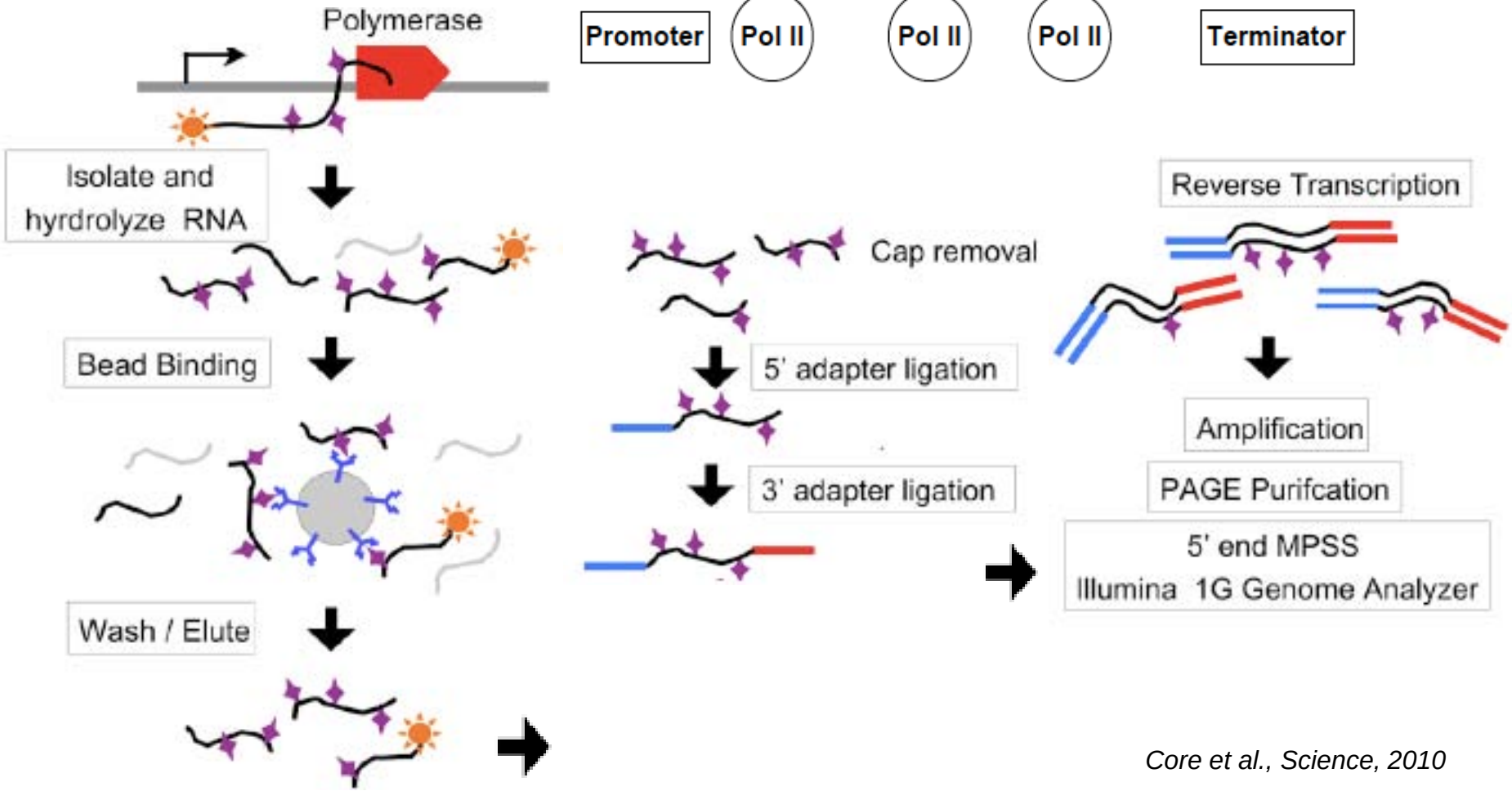
Method	Transcription step						
	TSS[a]	RNA capping	Promoter-proximal pausing	Co-transcriptional RNA processing	Transcription termination	Pol II CTD modification	Transcription bursting
Chromatin isolation-based methods							
caRNA-seq	No	No	No	Yes[42,105–107]	No	No	No
Start-seq	Yes[43]	No	Yes[43]	No	No	No	No
mNET-seq	No	No	Yes[41,73]	Yes[41,63,64]	Yes[41]	Yes[41,63]	No
SMIT-seq	No	No	No	Yes[159,160]	No	No	No
Run-on methods							
GRO-cap and PRO-cap	Yes[4,42]	No	No	No	No	No	No
GRO-seq, PRO-seq and ChRO–seq	No	No	Yes[42,48,74]	Yes[166]	Yes[42]	No	No
CoPRO	Yes[49]	Yes[49]	Yes[49]	No	No	No	No
Metabolic labelling methods							
TT-seq	No	No	No	No	Yes[47]	No	No
Imaging-based methods							
Intron sequential FISH	No	No	No	No	No	No	Yes[55]

Wissink et al, Nat Rev Genet, 2019

Analysis of Nascent Transcripts- GRO-seq



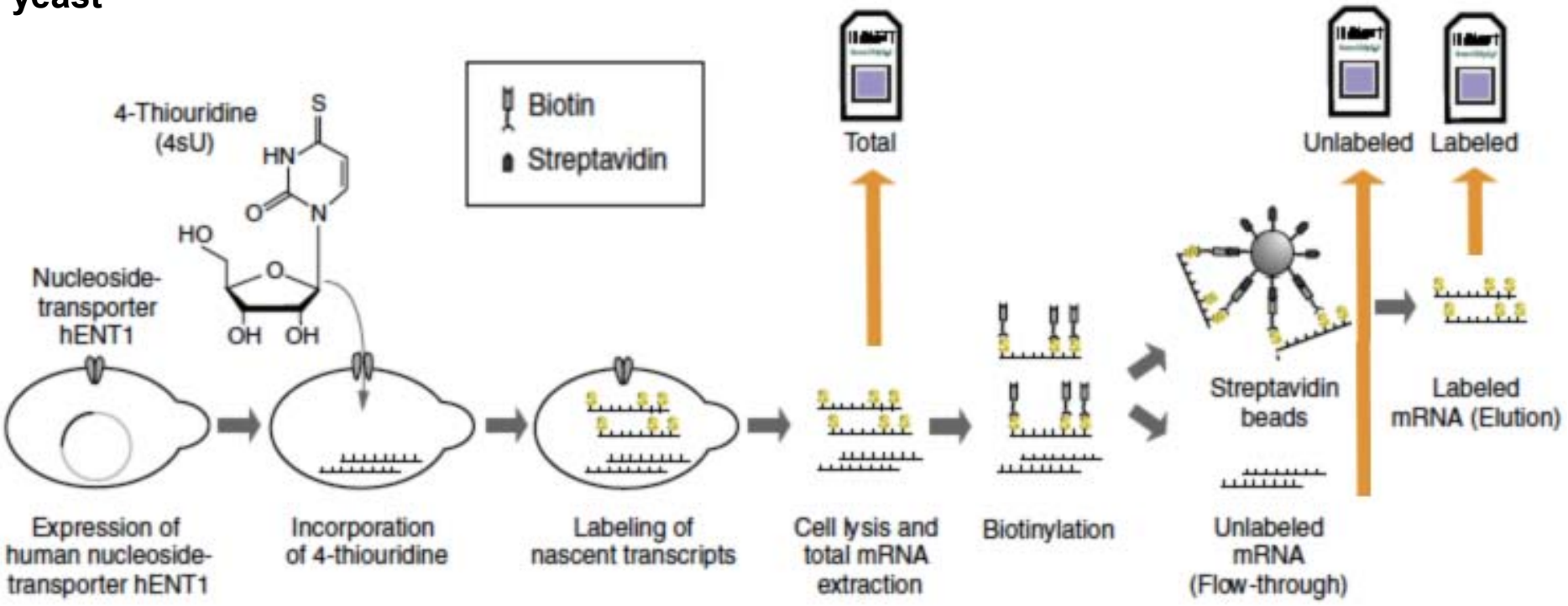




Core et al., Science, 2010

Analysis of Nascent Transcripts

yeast



Expression of hENT1 nucleoside transporter enables uptake of UTP derivatives

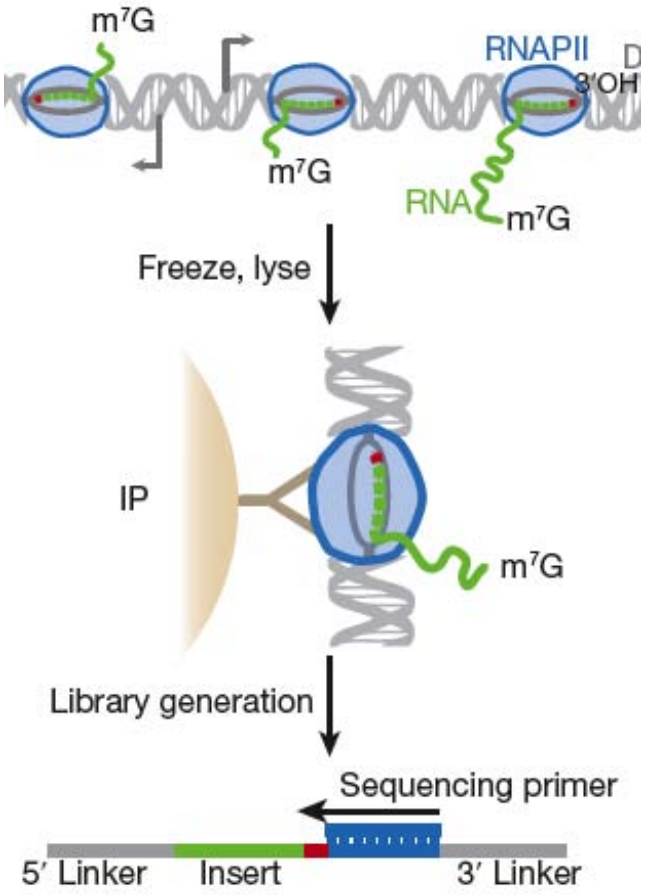
Non-perturbing RNA labeling in yeast
Allows dynamic transcriptome analysis: sythesis and decay rates and the study of nascent transcripts

Miller et al., Mol Syst Biol, 2010;
Barrass et al, Genome Biol, 2015

Analysis of Nascent Transcripts

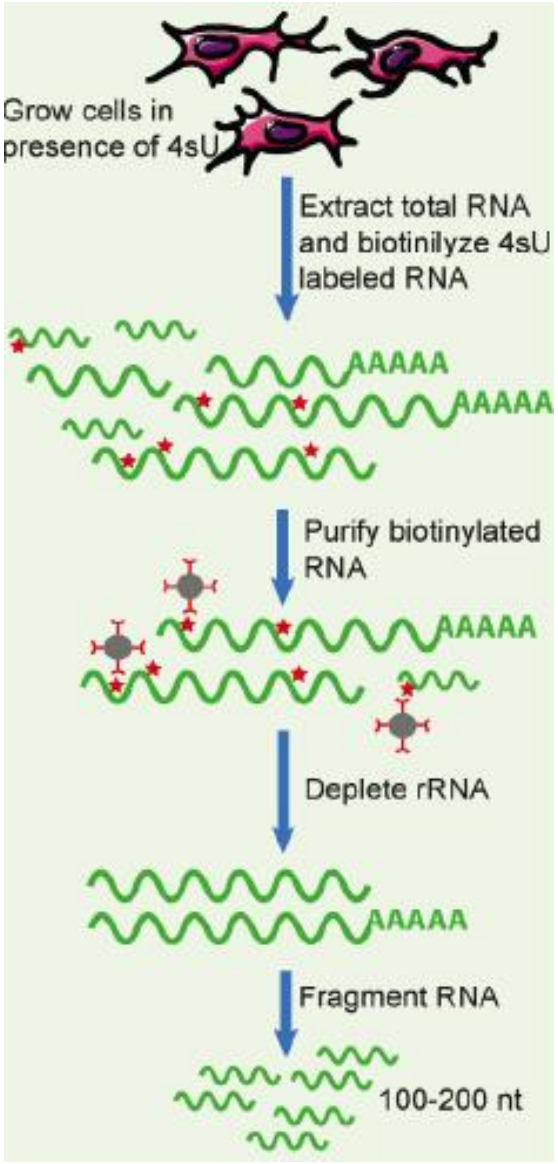
I.

NET-Seq



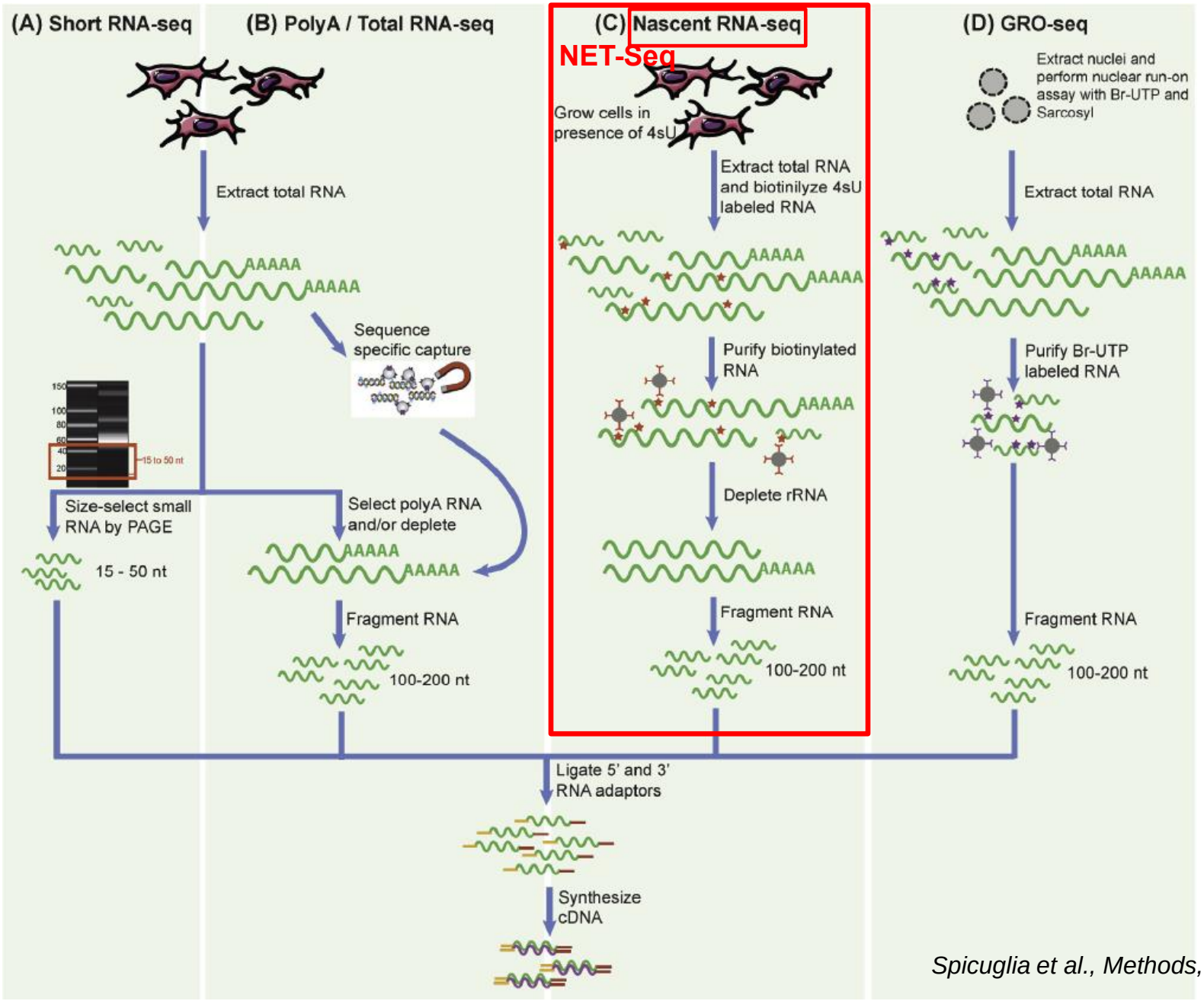
Churchman and Weissman, Nature, 2011

II.



Spicuglia et al., Methods, 2013

Comparison of different RNA-Seq approaches

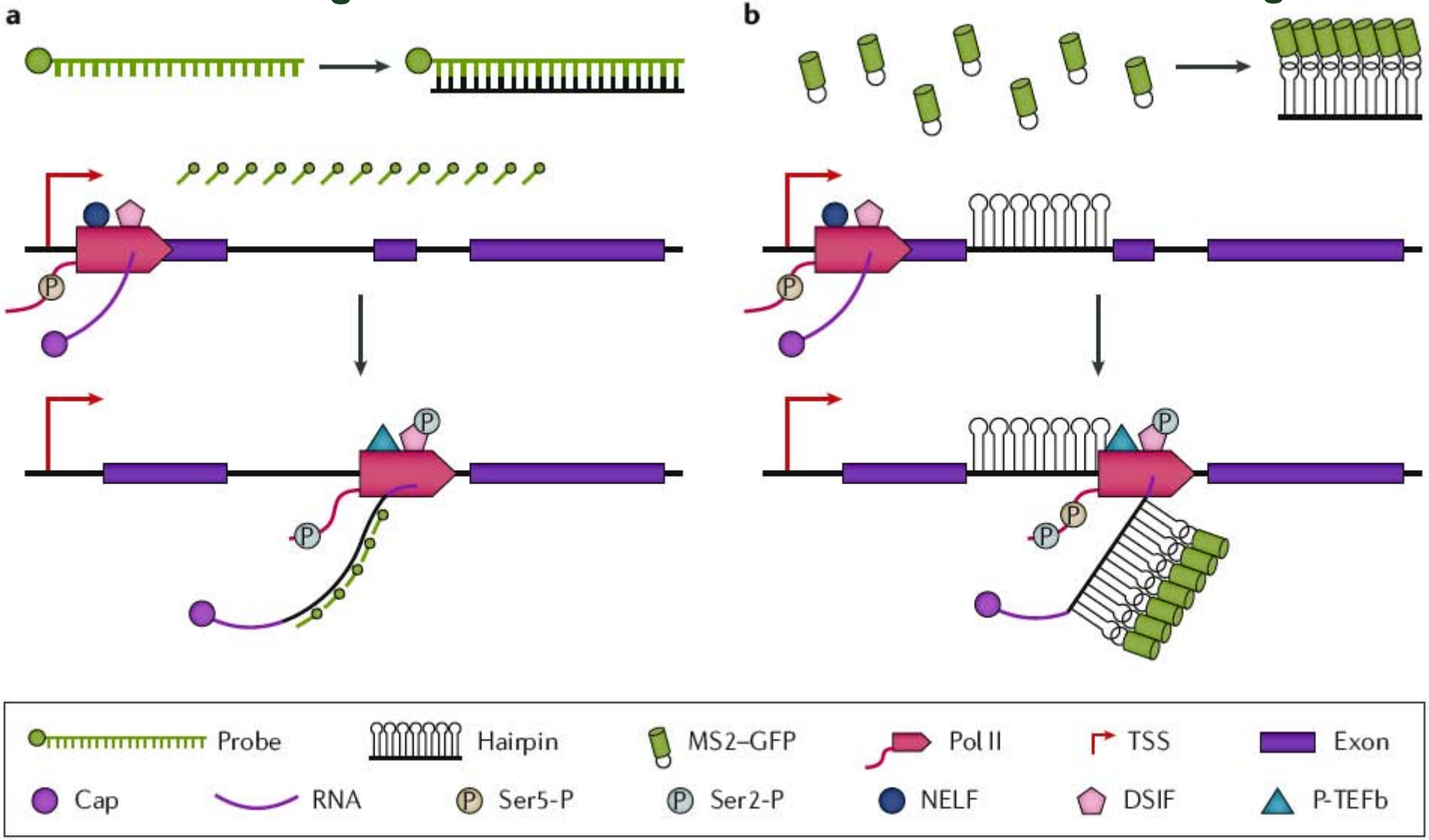


Spicuglia et al., Methods, 2013

Imaging of nascent RNA

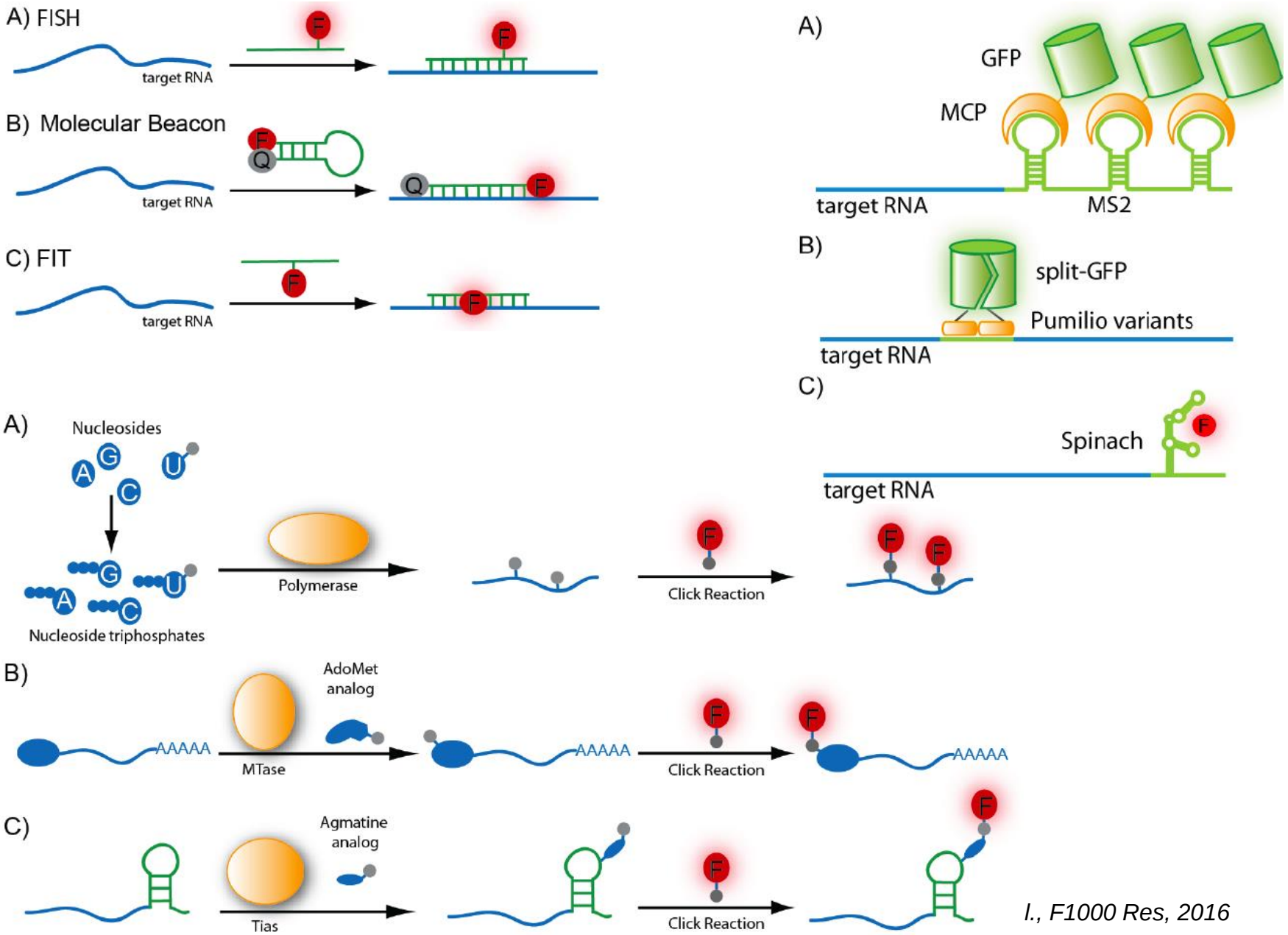
smFISH – single molecules FISH

in living cells
MS2/MCP labeling



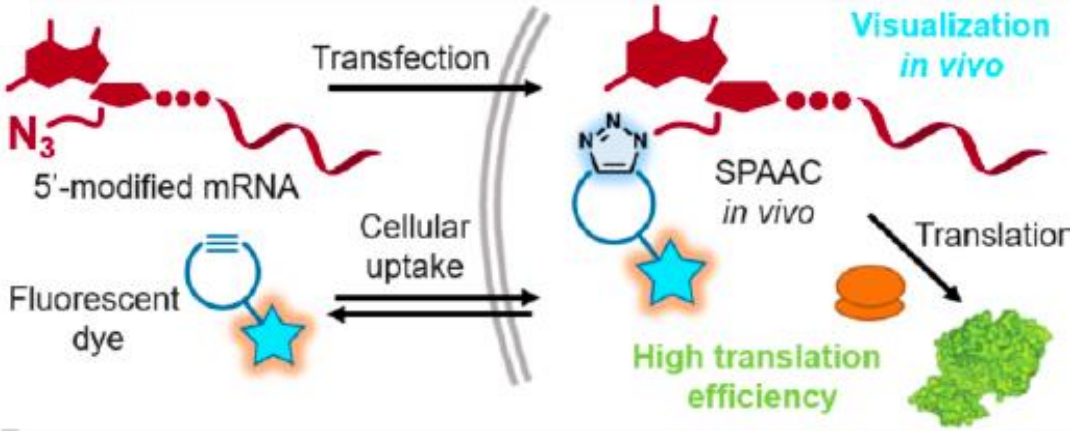
Wissink et al, Nat Rev Genet, 2019

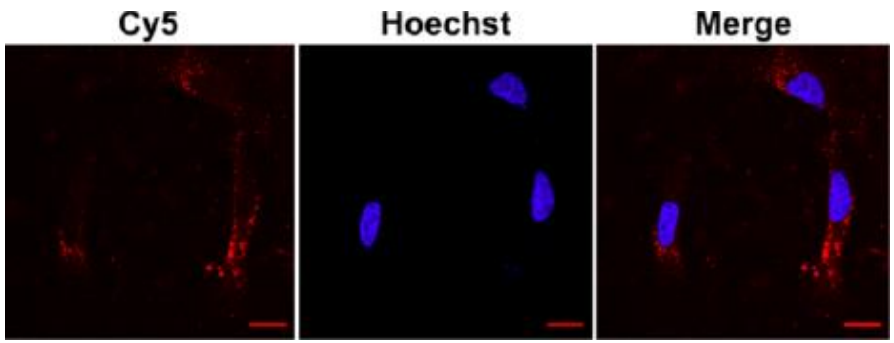
mRNA fluorescent labeling for imaging

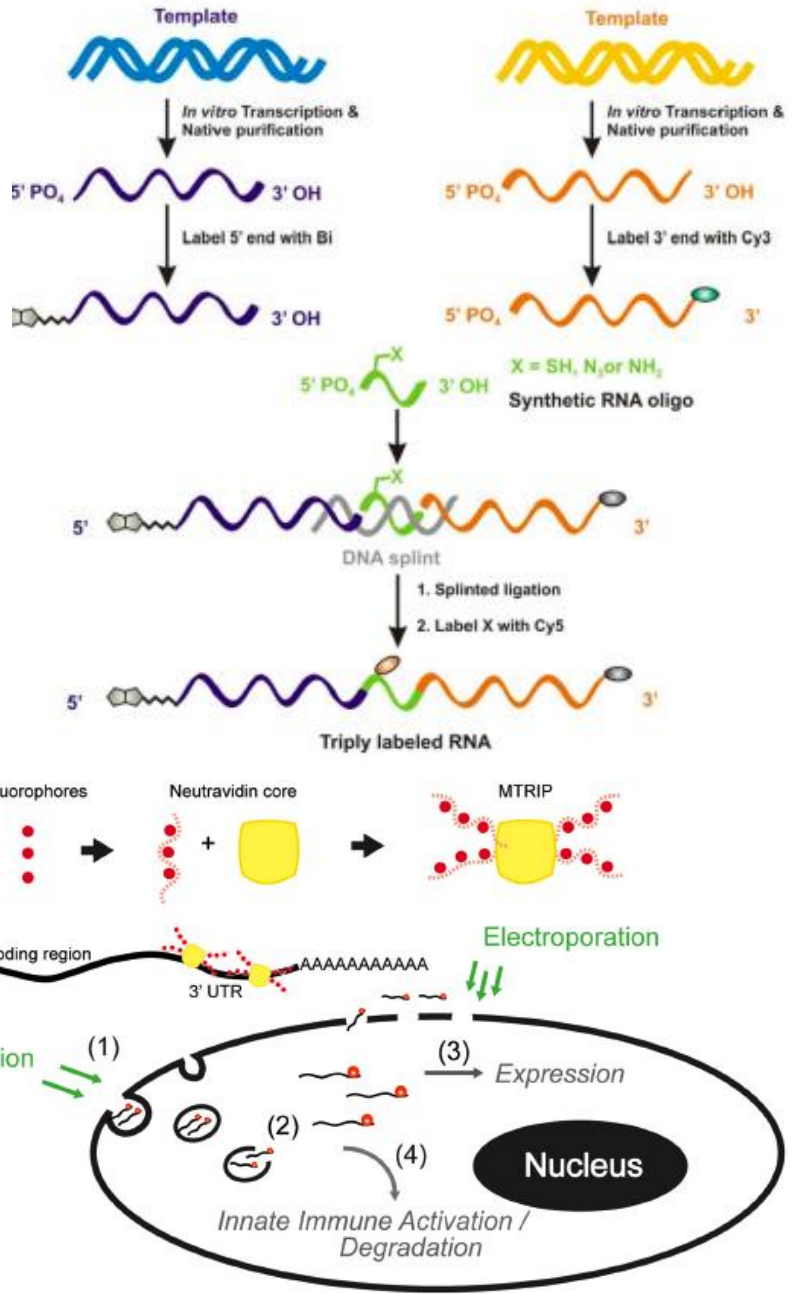


I., F1000 Res, 2016

mRNA fluorescent labeling for imaging

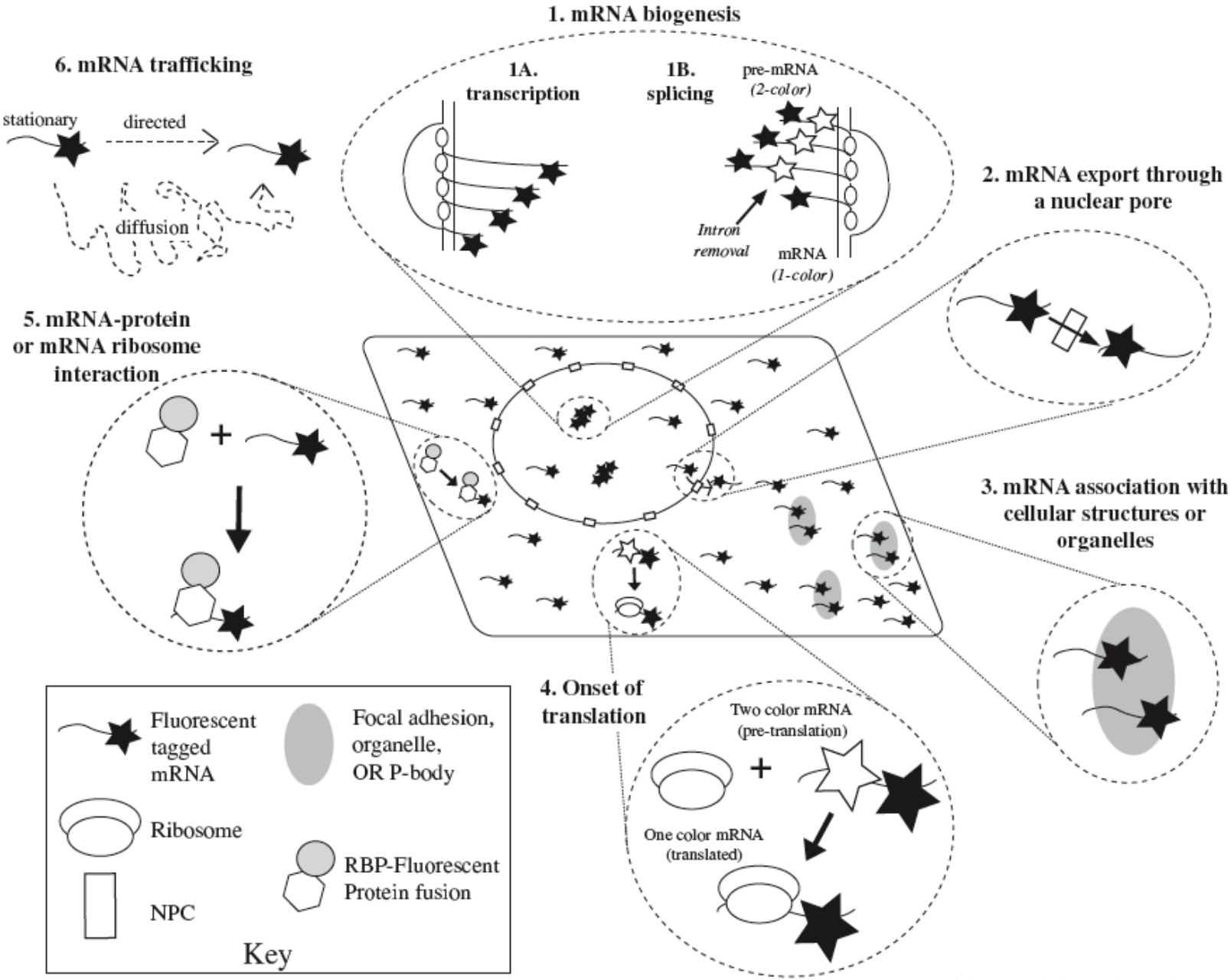






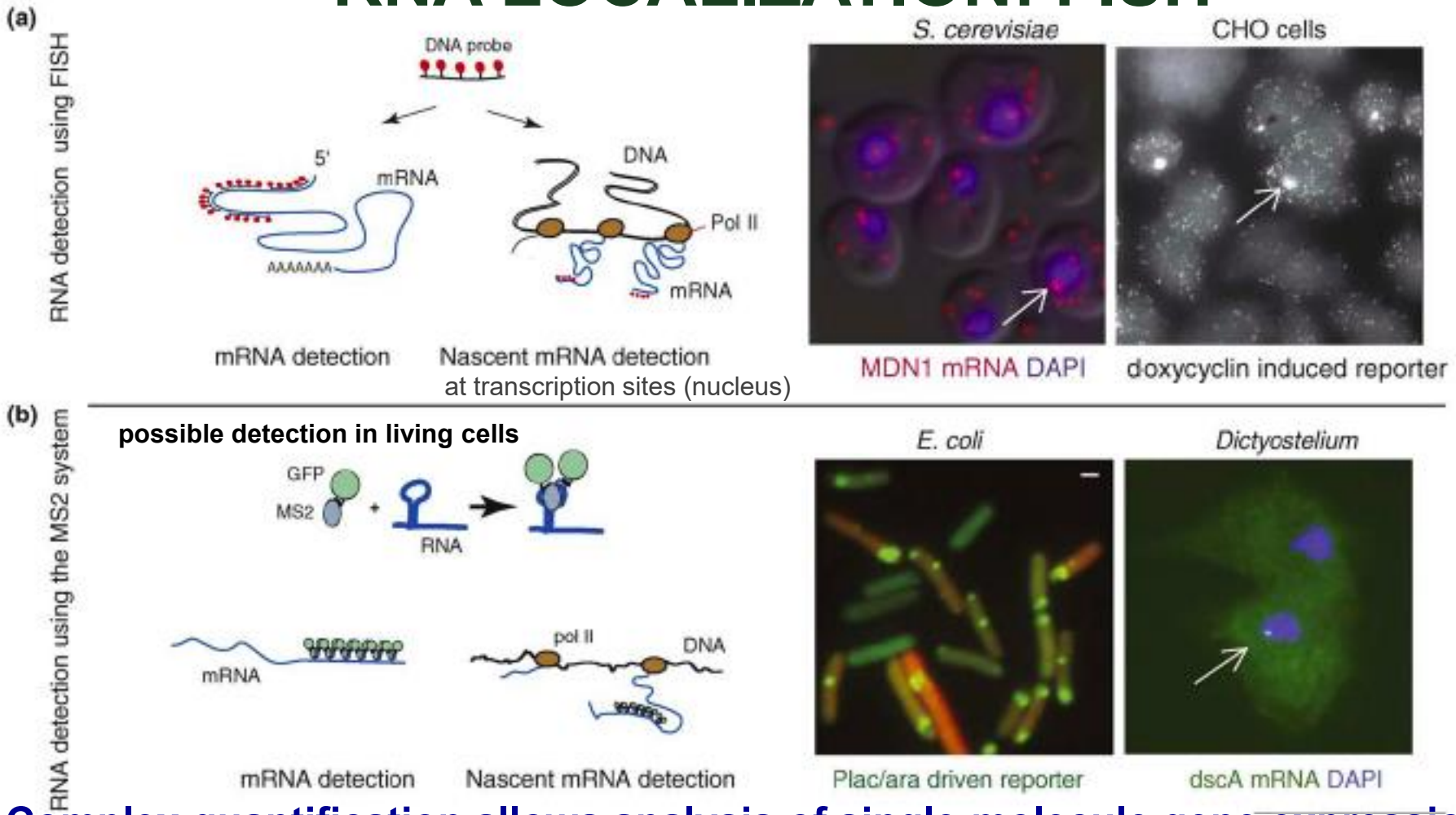
Mamot et al, Angew Chem, 2017
Rinaldi et al., Methods Mol Biol, 2015
Kirschman et al. NAR 2017

FISH: Fluorescent *in situ* hybridization



Abbaszadeh and Gavis, Methods, 2016

RNA LOCALIZATION: FISH



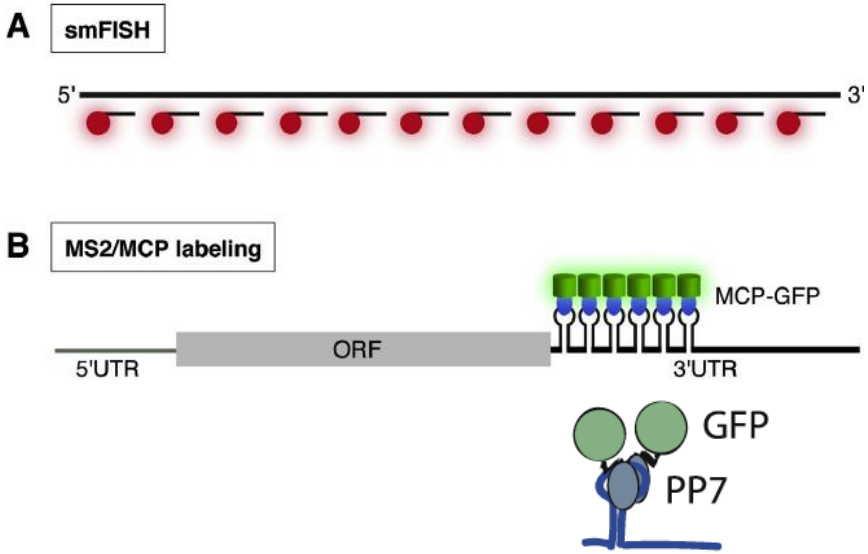
Larson et al., TiCB, 2009

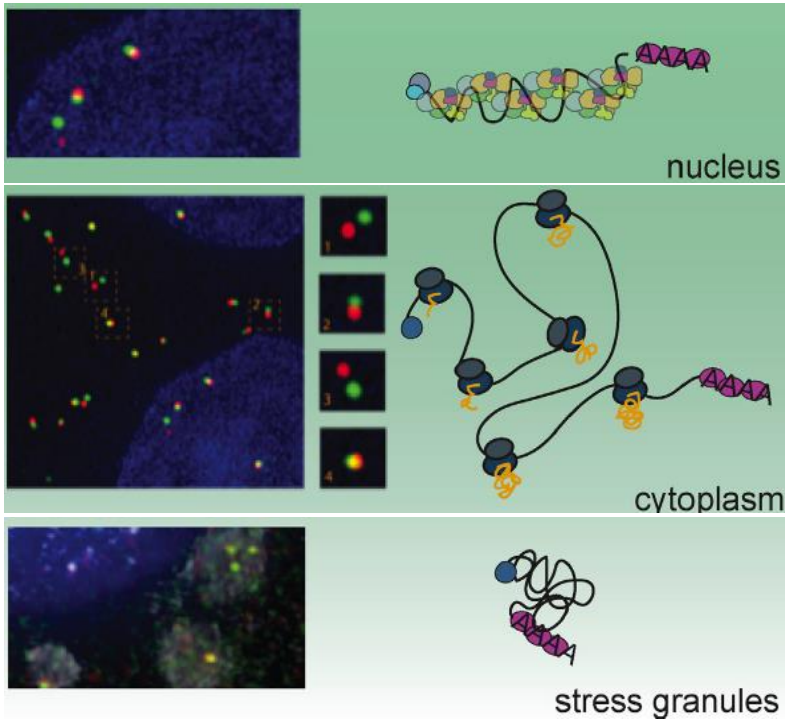
Complex quantification allows analysis of single-molecule gene expression, e.g. transcription/splicing in real time, RNA level in single cells.

- **Constitutively expressed genes are transcribed by single events separated in time; regulated genes (e.g. by SAGA) are expressed by transcriptional bursts**
- **Transcription of functionally related constitutive genes is not coordinated**

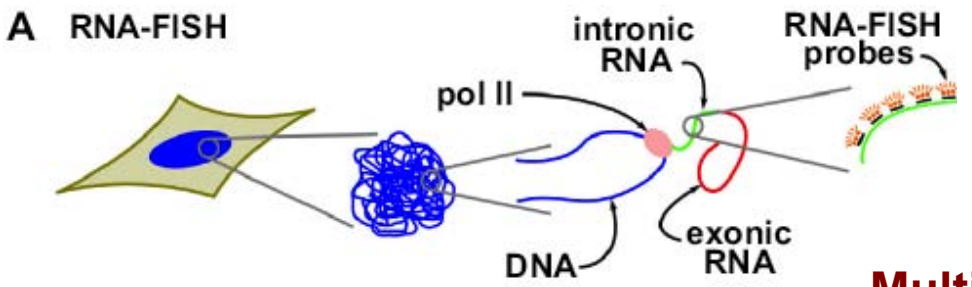
(regulated post-transcriptionally or post-translationally) */Singer lab/*

RNA LOCALIZATION: FISH

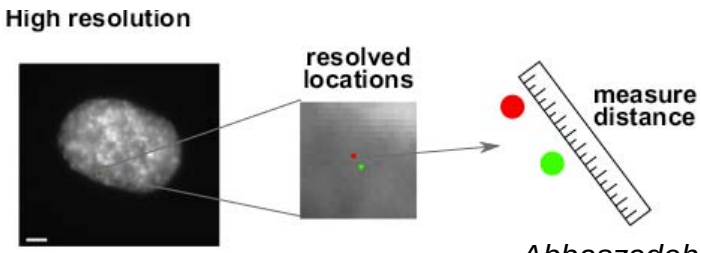




Adivarahan et al., Mol Cell 2018



RNA detection at transcription sites with intronic probes



Multi-colored smFISH:

1. Nuclear mRNAs are partially extended

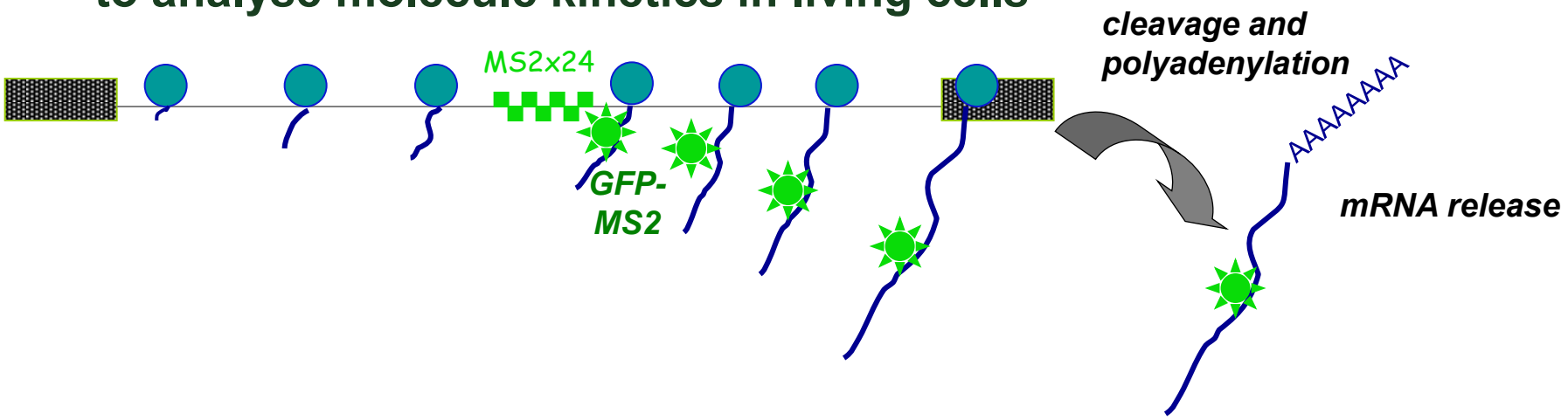
2. Translating mRNAs usually do not have a circular form (no interacting 5' and 3' ends)

3. mRNAs in stress granules are more compacted than translating mRNAs

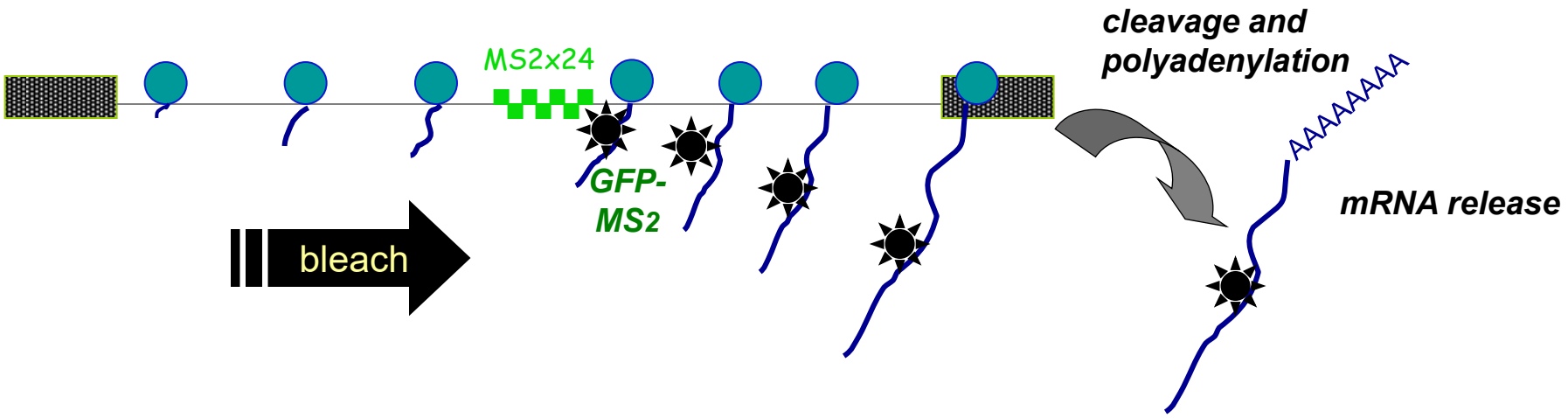
Abbaszadeh and Gavis, Methods, 2016

RNA LOCALIZATION: FRAP and FLIP

FRAP - fluorescence recovery after photobleaching
FLIP - fluorescence loss in photobleaching
to analyse molecule kinetics in living cells



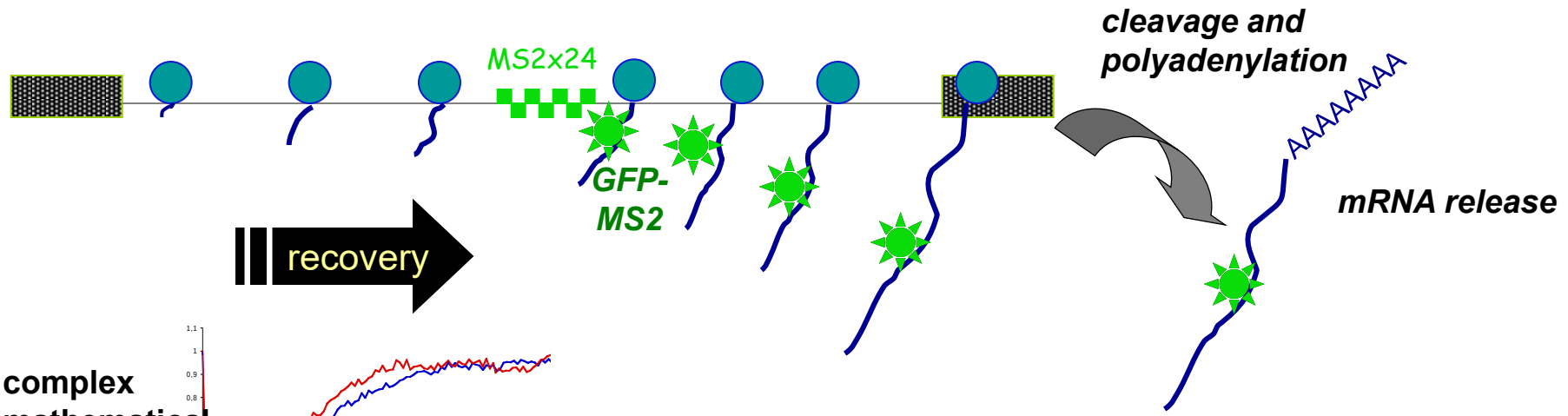
RNA LOCALIZATION: FRAP



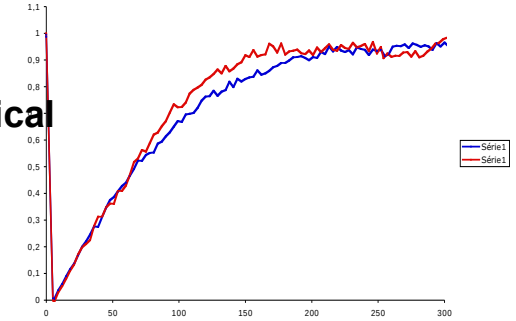
Edouard Bertrand, Montpellier, *RIBOSYS*

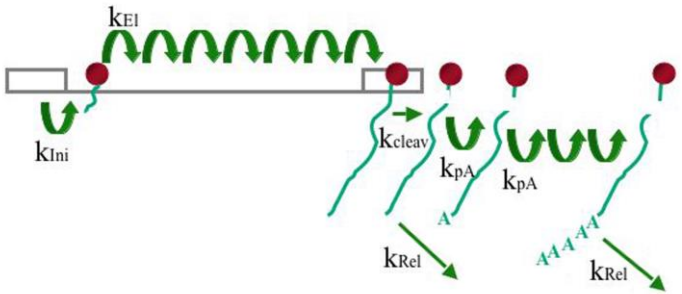
RNA LOCALIZATION: FRAP

Analysis of: - transcription rates
- 3'-end formation
- transcript release

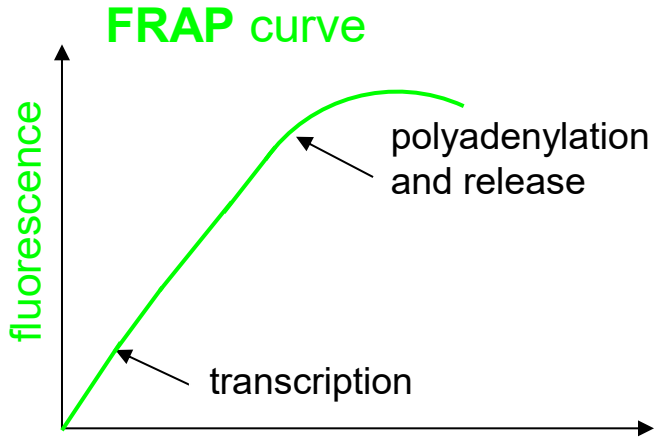


complex mathematical modeling



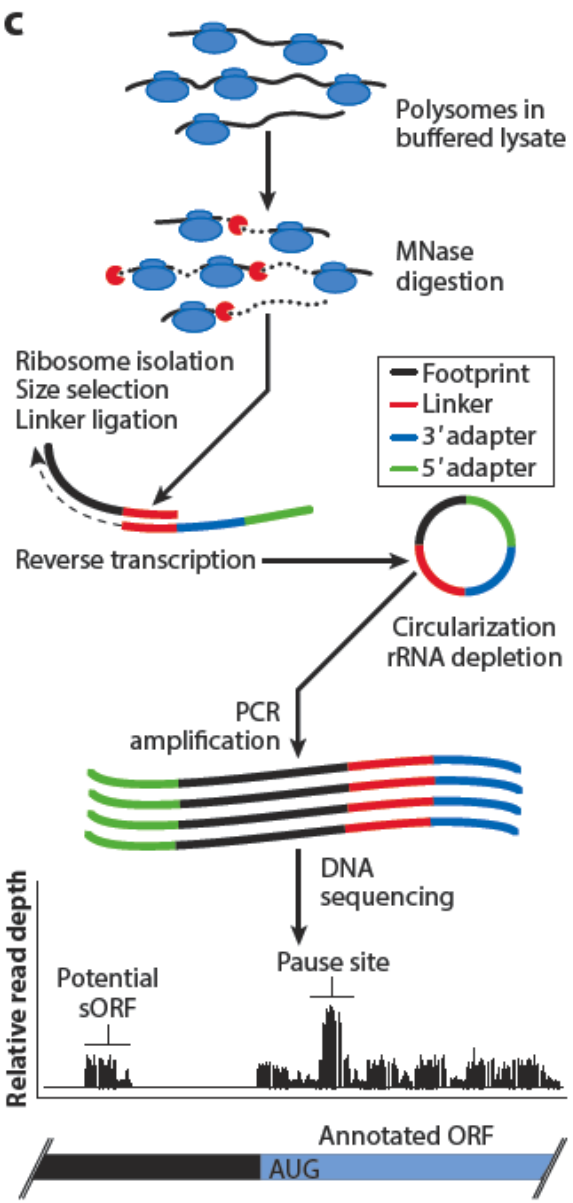


trx longation rate: 2 kb/min



Edouard Bertrand, Montpellier, *RIBOSYS*

Other modern approaches



Ribosome Profiling
Ribosome Mapping
Ribo-seq

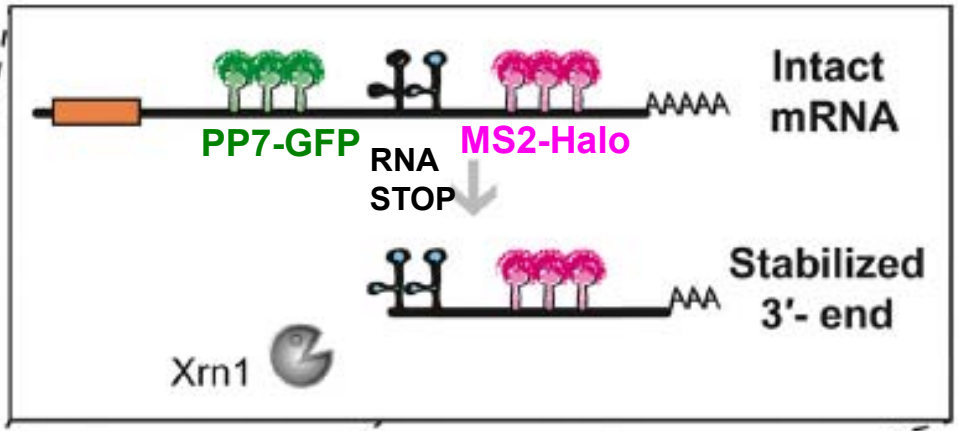
In vivo analysis of translating ribosomes and mRNAs

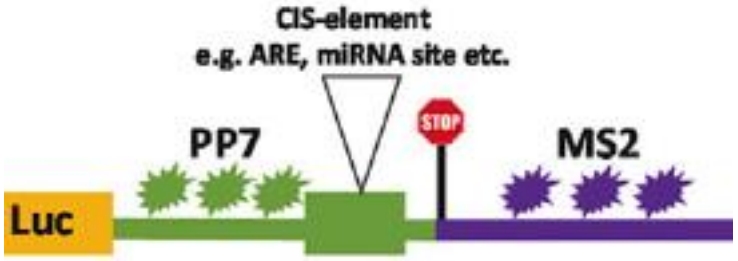
- transcript abundancy
- translation kinetics and efficiency/protein synthesis rate
- polysome occupancy profiles
- co-translational processes
- combined with RNASeq- correlations between transcription and translation
- short and alternative ORFs

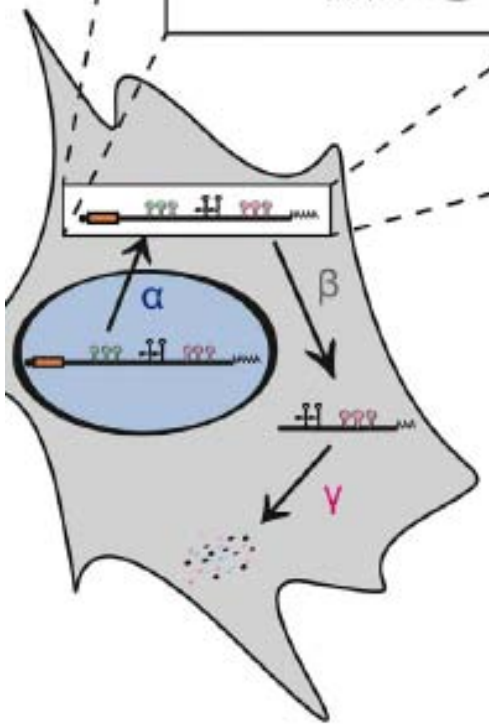
Barquist and Vogel, *Ann Rev Genet, 2015*

TREAT- 3'-RNA end accumulation during turnover

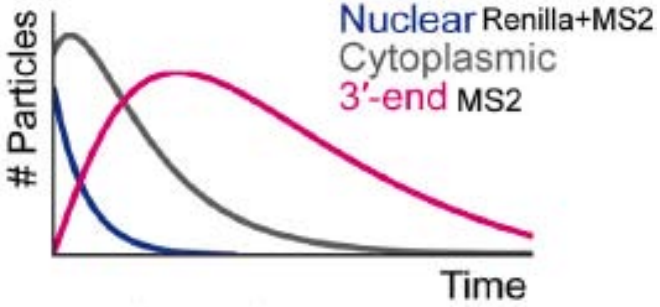
Single-mRNA imaging of RNA degradation in single cells







Real-time imaging of siRNA-mediated slicing

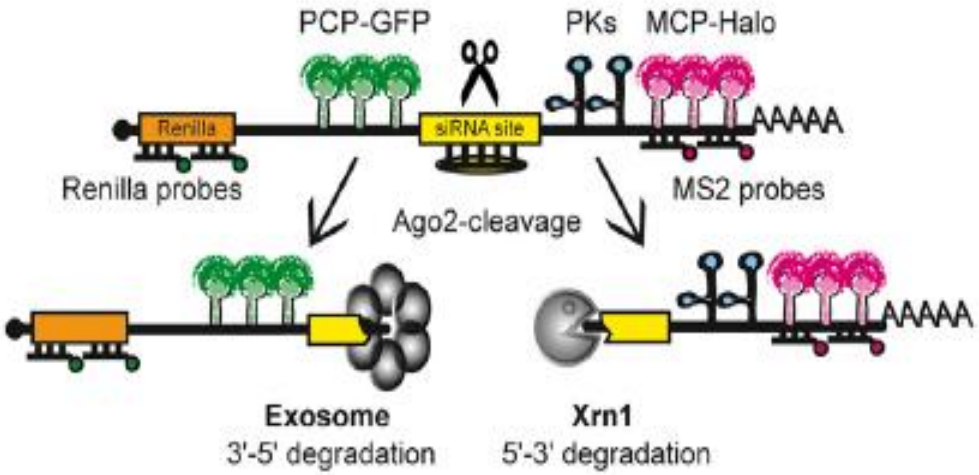


Quantification of mRNA decay dynamics

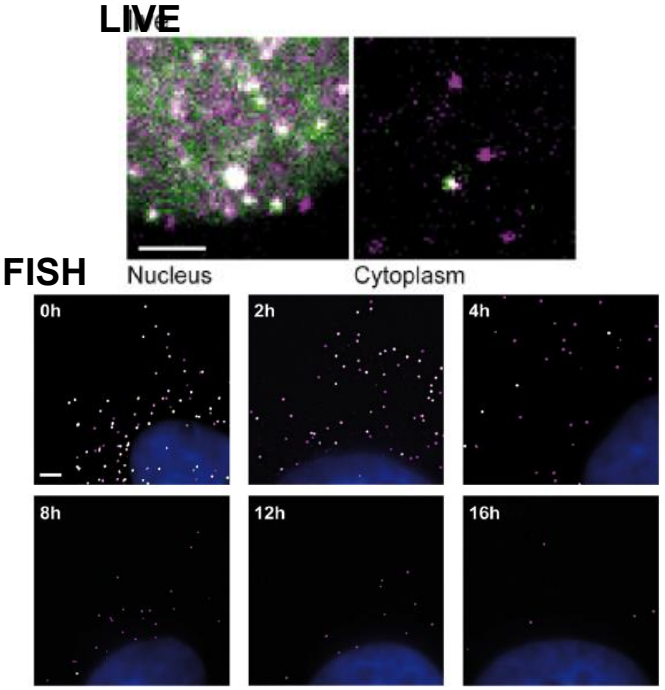
Horvathova et al, Mol Cell, 2017

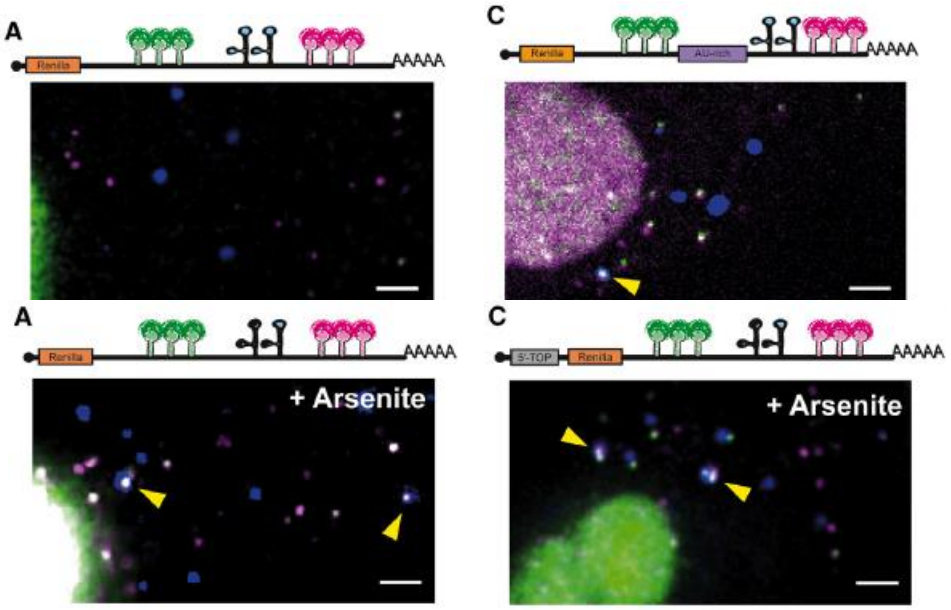
TREAT- 3'-RNA end accumulation during turnover

- **real-time observation of Ago2 slicing of TREAT mRNAs**



- **single-mRNA imaging of RNA degradation in single cells**



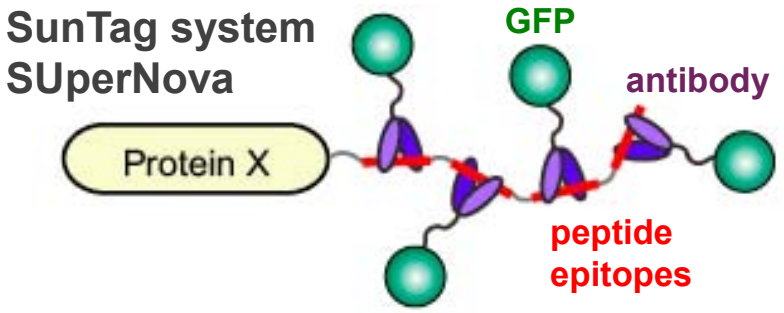


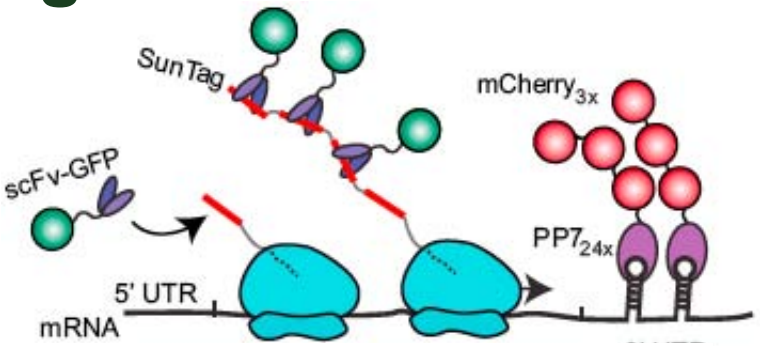
- **TREAT mRNAs are not degraded in P-bodies, also in stress**

DCP1- PB marker

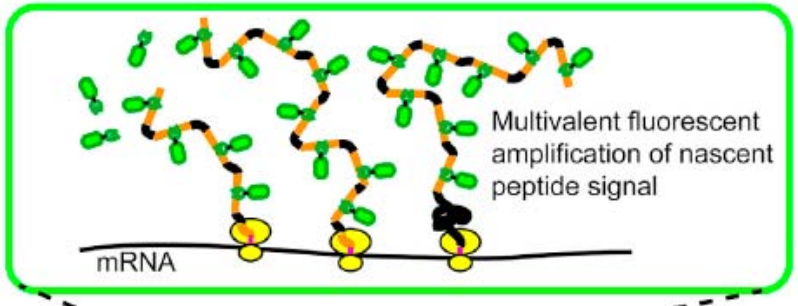
Horvathova et al, Mol Cell, 2017

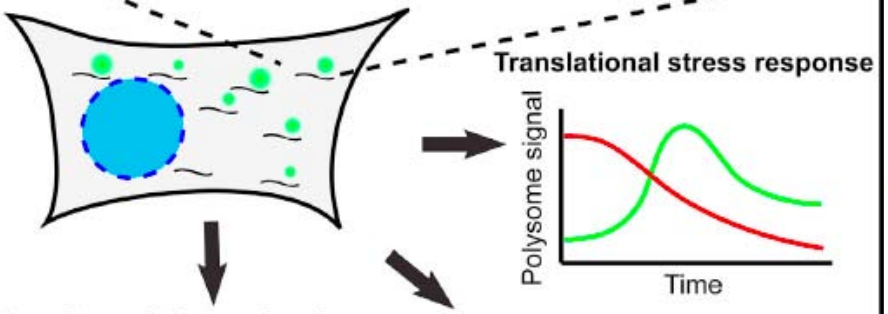
SunTag -Translation of single mRNAs *in vivo*





Translation visualized by labeling nascent peptide epitopes with antibody-GFP

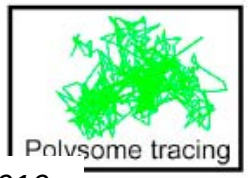


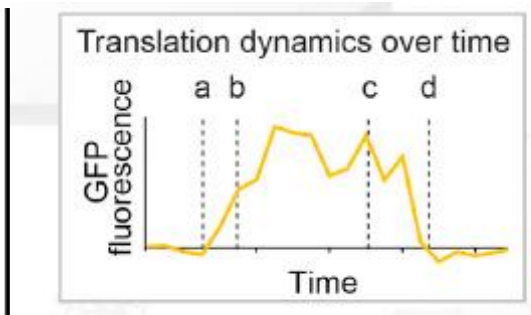


Translational stress response

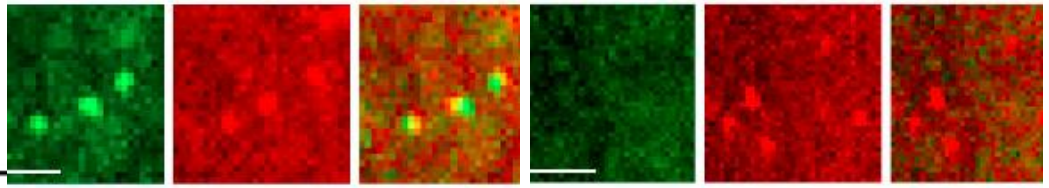
Local translation and active transport of polysomes

Polysome mobility analysis



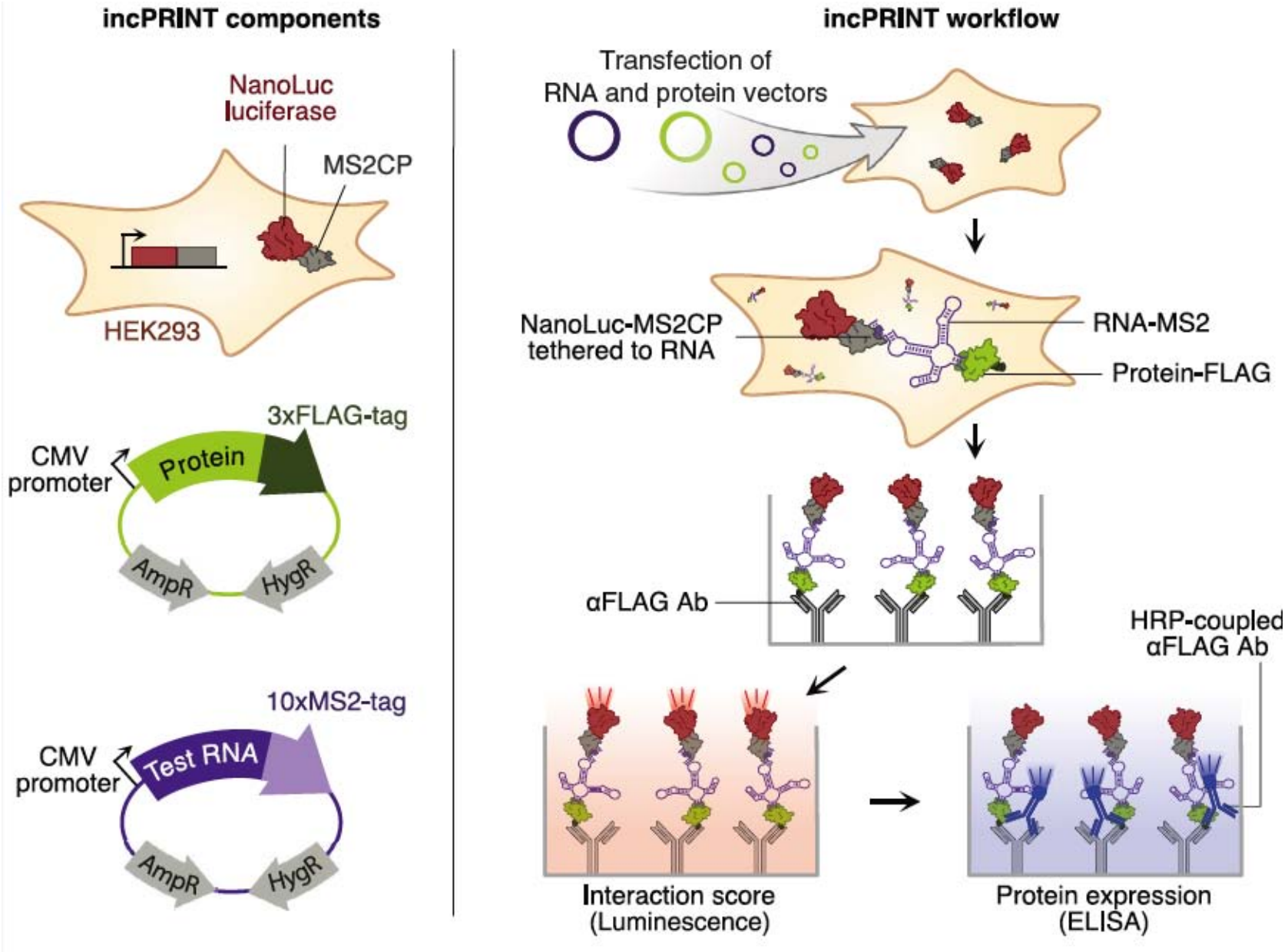


mCherry-labeled mRNA tethered to the membrane



Wang et al, Cell, 2016 Yan et al., Cell 2016

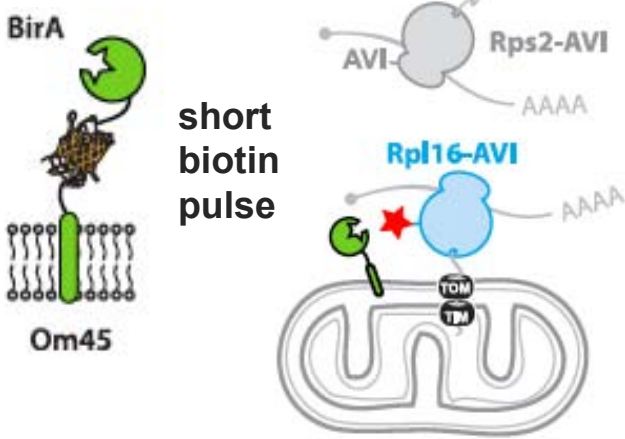
In-Cell Discovery of RNA–Protein Interactions
incPRINT

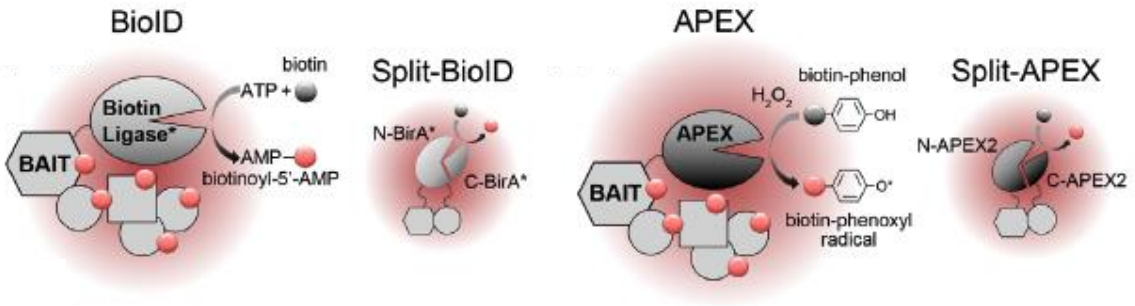


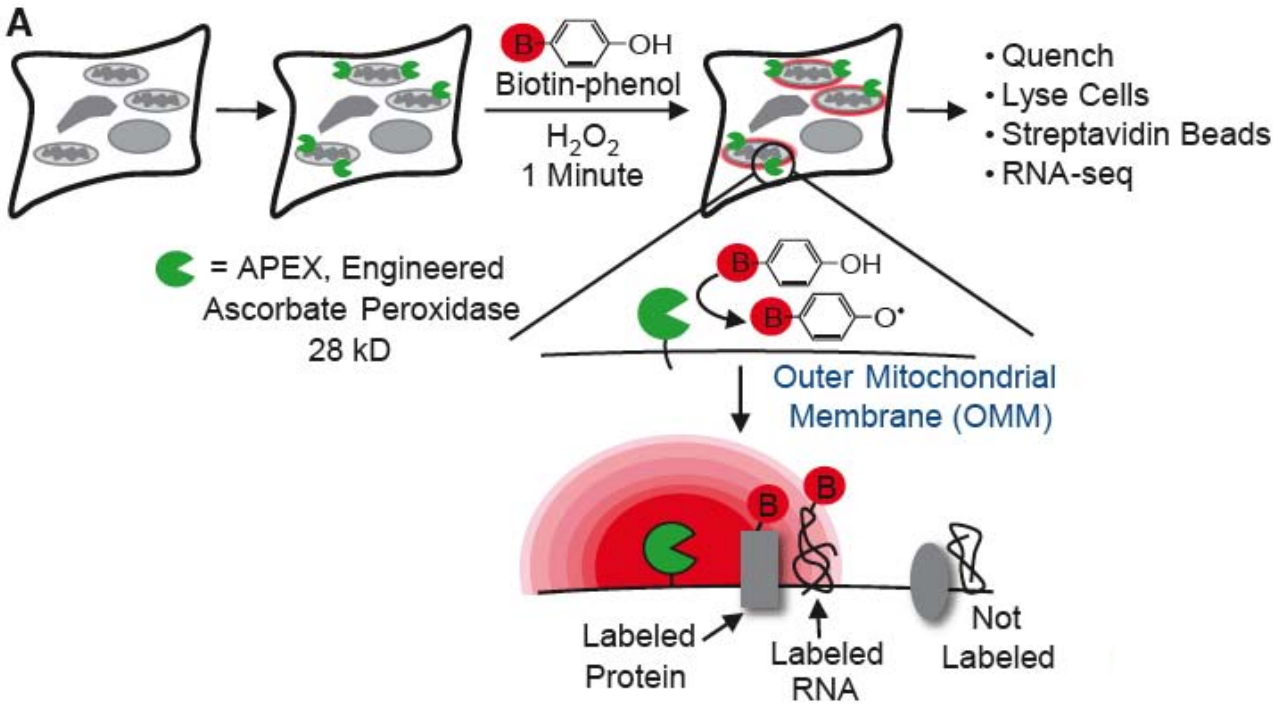
Sabaté-Cadenas and Shkumatava, TiBS'20

APEX and BioID

Proximity biotin-based labeling methods





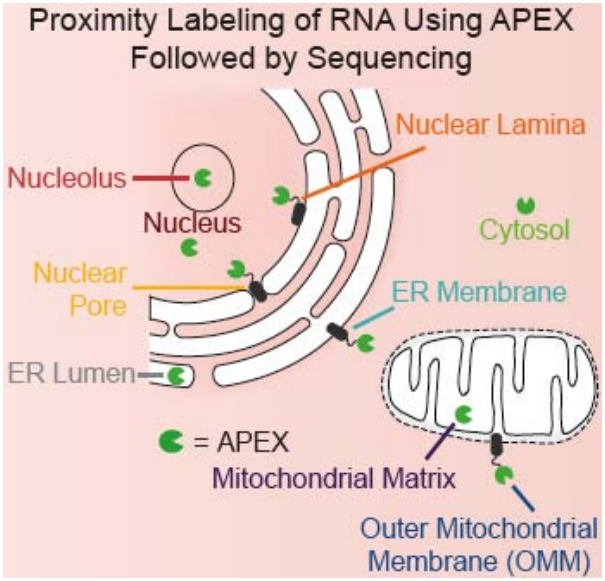


Tag	Enzyme Activity	Source
BirA*	Biotin Ligase (promiscuous)	*E. Coli*
TurboID	Biotin Ligase (promiscuous)	*E. Coli*
MiniTurbo	Biotin Ligase (promiscuous)	*E. Coli*
BioID2	Biotin Ligase (promiscuous)	*A. Aeolicus*
BASU	Biotin Ligase (promiscuous)	*B. Subtilis*
APEX	Ascorbate peroxidase	Pea (synthetic)
APEX2	Ascorbate peroxidase	Soybean (synthetic)

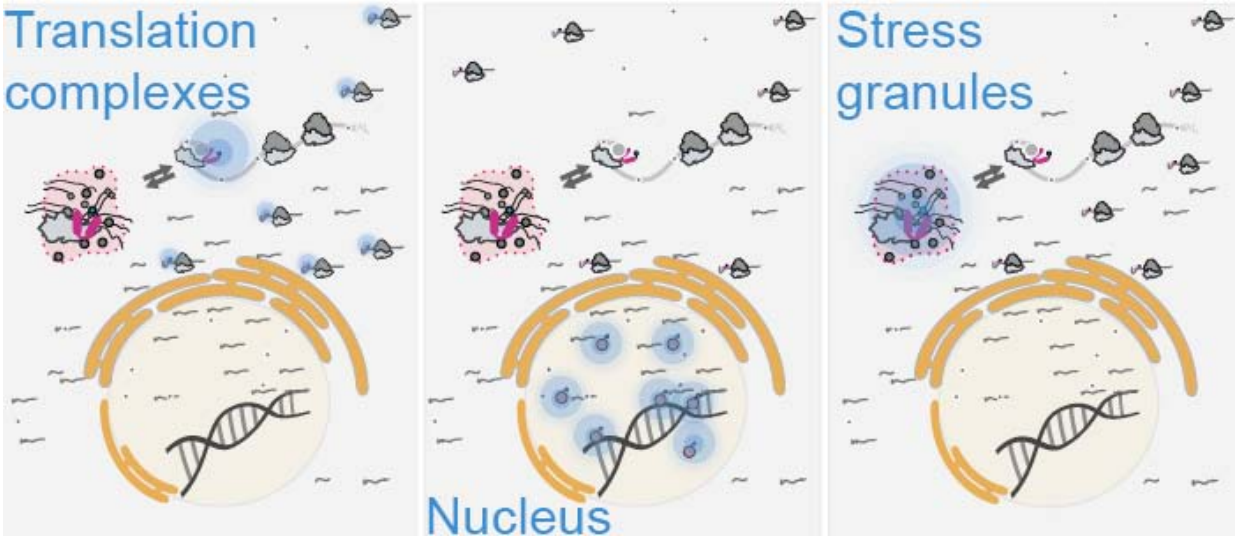
Williams et al, Science, 2014; Fazal et al, Cell, 2019; Trinkle-Mulcahy, F1000Research, 2020

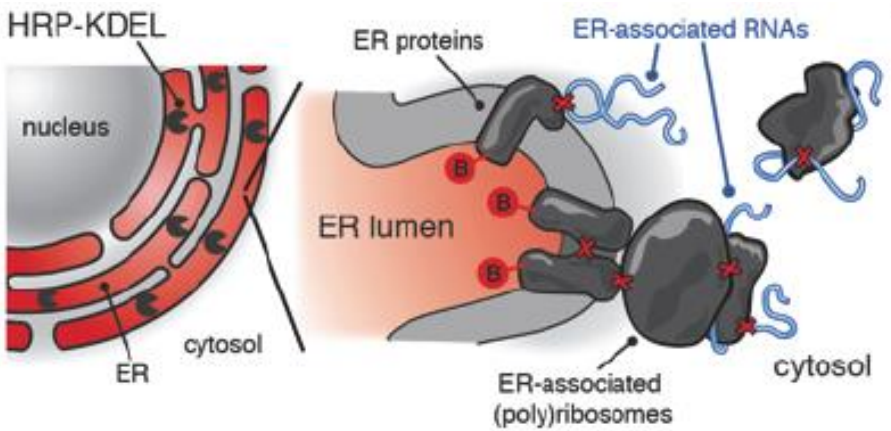
APEX: applications

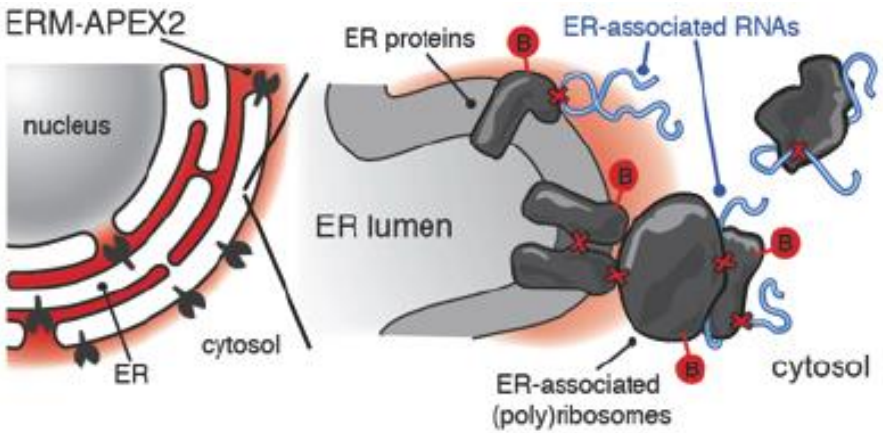
Protein and RNA localization



RNP organization- translation, granules



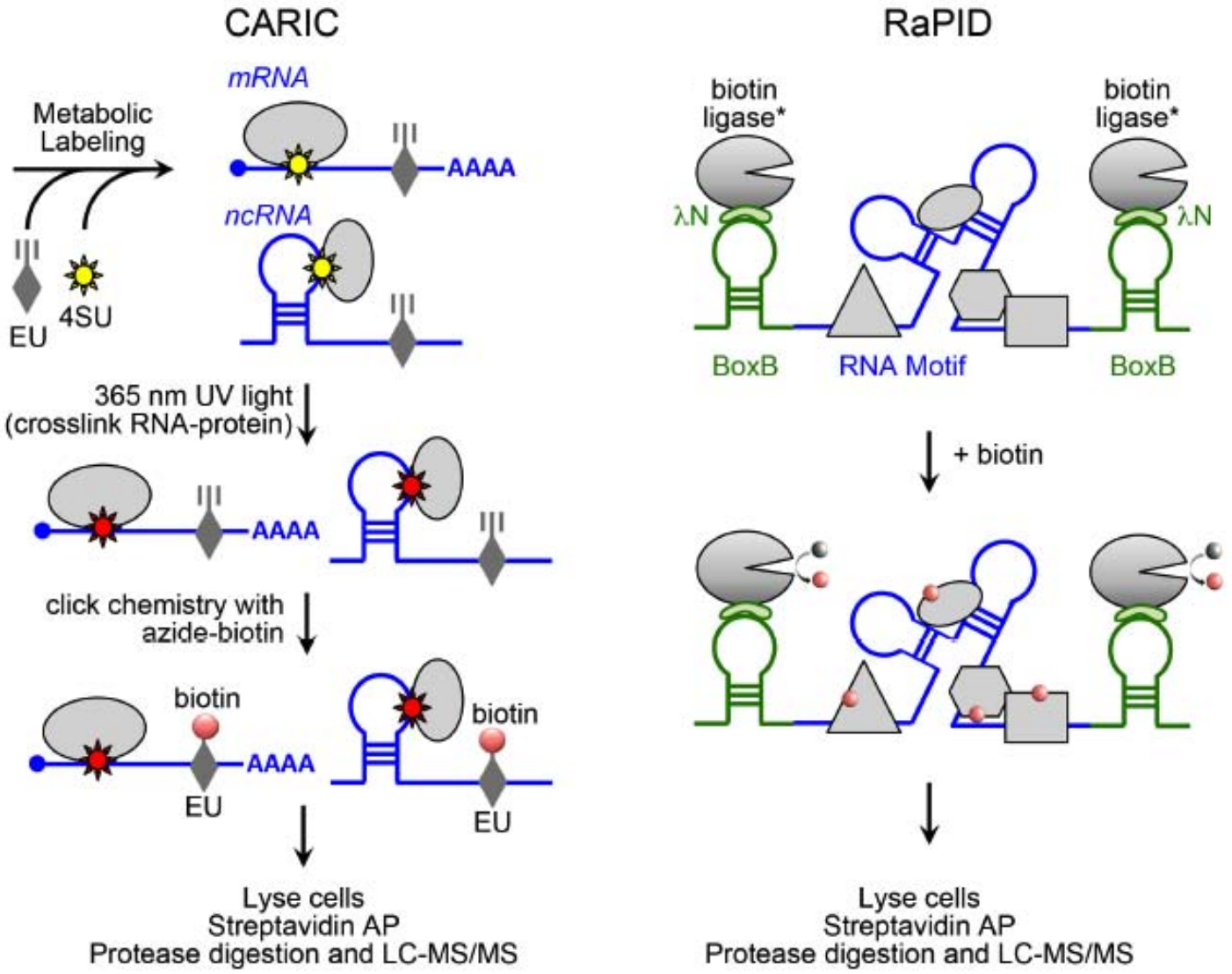




Fazal et al, Cell, 2019; Padron et al, Mol Cell, 2019

APEX: applications

Protein-RNA (RNP) complexes

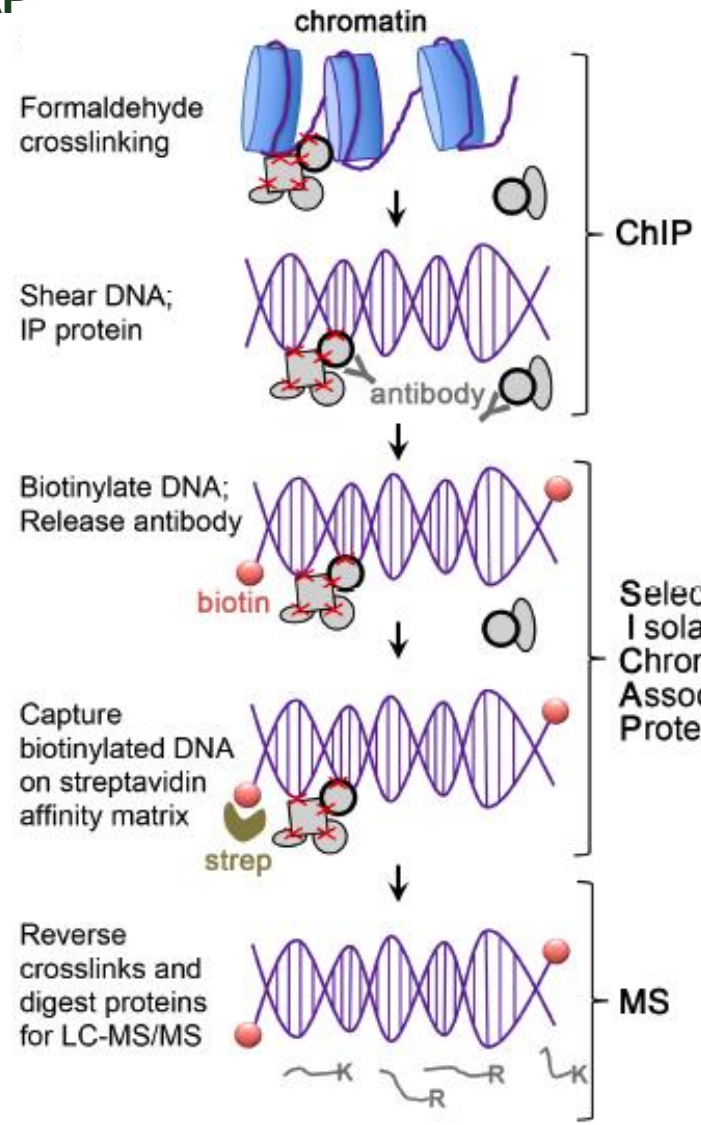


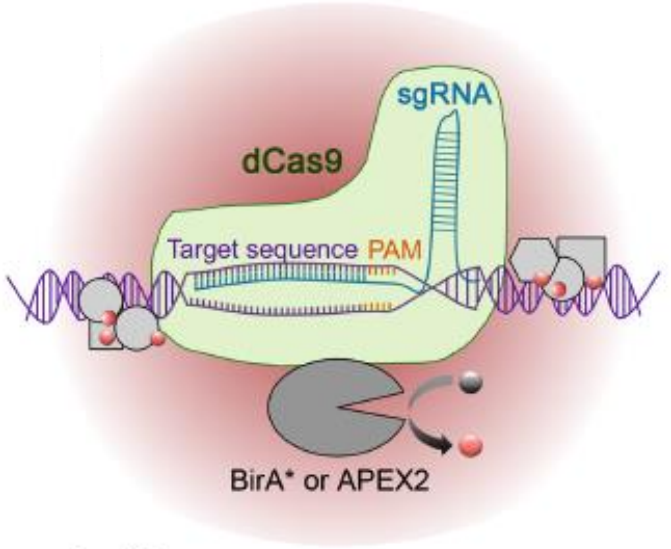
Trinkle-Mulcahy, F1000Research, 2020

APEX: applications

Chromatin-associated protein complexes

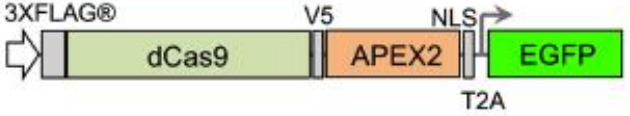
ChIP-SICAP



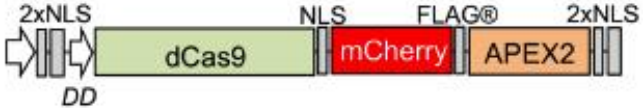


CasID

CASPEX



C-BERST



Trinkle-Mulcahy, F1000Research, 2020

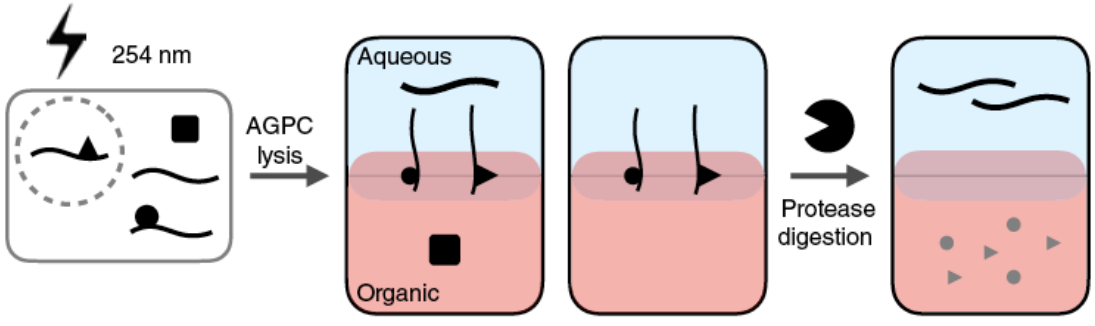
OOPS, TRAPP, XRNAX RNP interactome, RPBome

OOPS - orthogonal organic phase separation

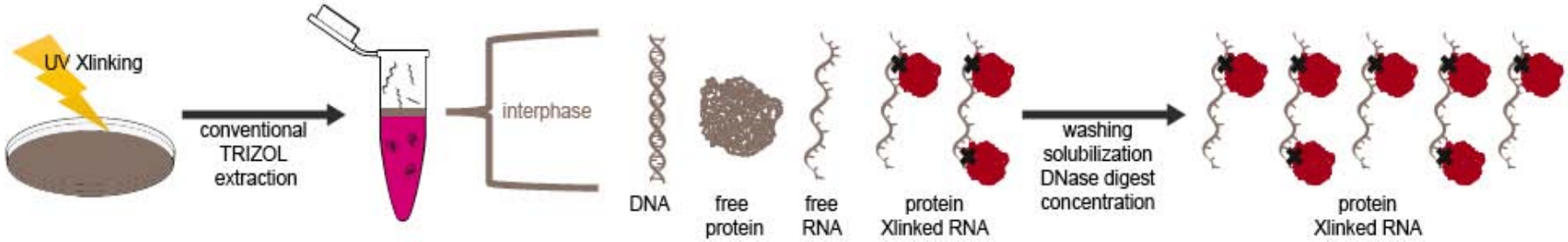
TRAPP/PAR-TRAPP - RNA-associated protein purification

XRNAX

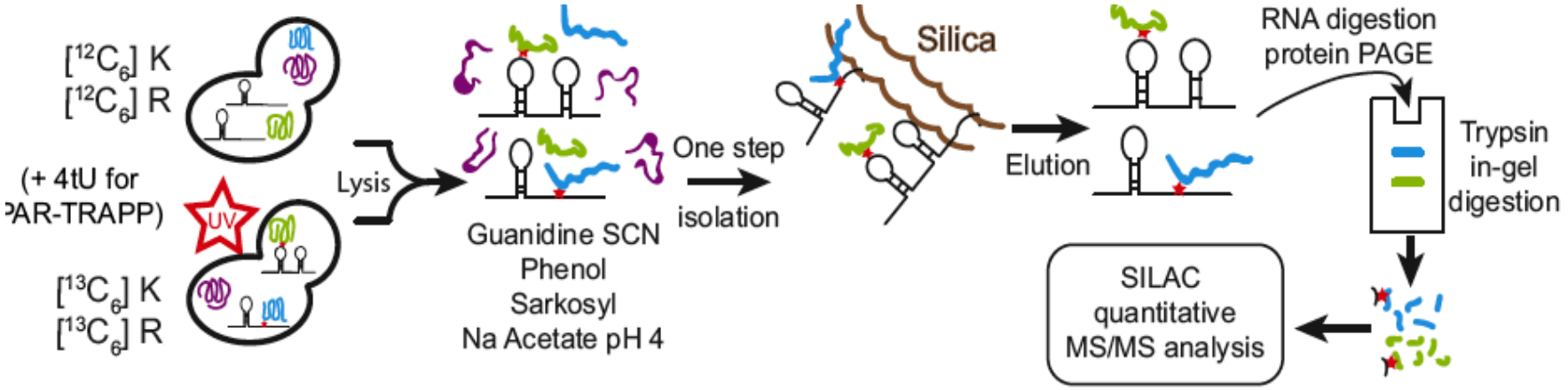
OOPS



XRNAX



TRAPP/PAR-TRAPP



Queiroz et al, Nat Biotech, 2019; Shchepachev et al, Mol Sys Biol, 2019

SELEX = Systematic Evolution of Ligands by EXponential enrichment

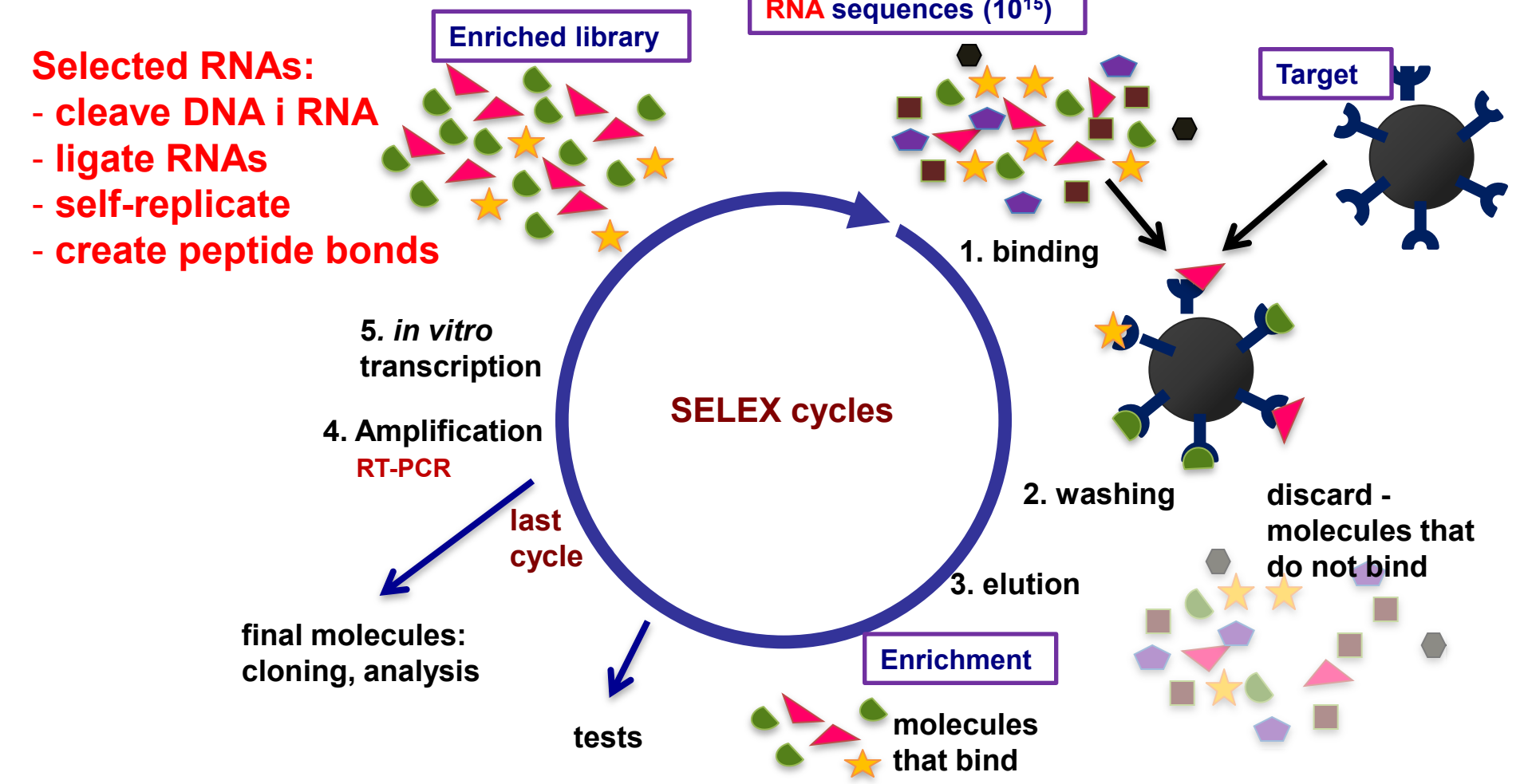
1990


Szostak


Gold

Method of selecting RNA/DNA molecules with desired properties (aptamers, ribozymes) based on cycles of amplification

Selected RNAs:
- cleave DNA i RNA
- ligate RNAs
- self-replicate
- create peptide bonds



SUMMARY or HOW TO PASS THE EXAM?

- THEORY
- METHODS
- GENERAL IDEAS, CONCEPTS, SOLUTIONS
- PATHWAYS
- MECHANISMS