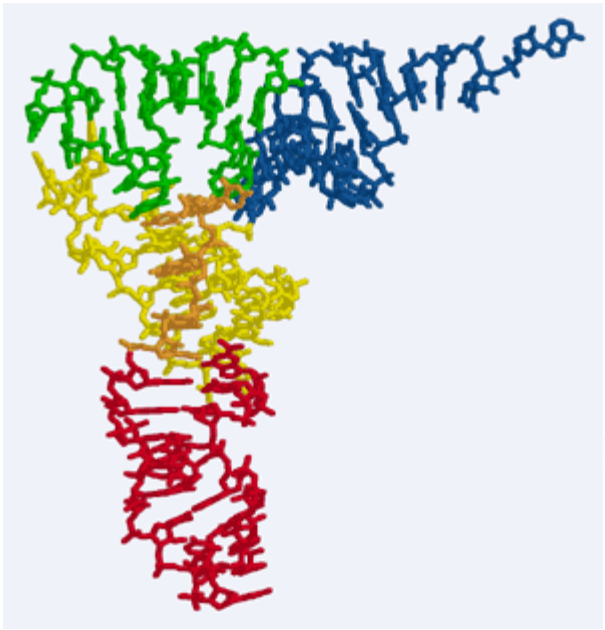
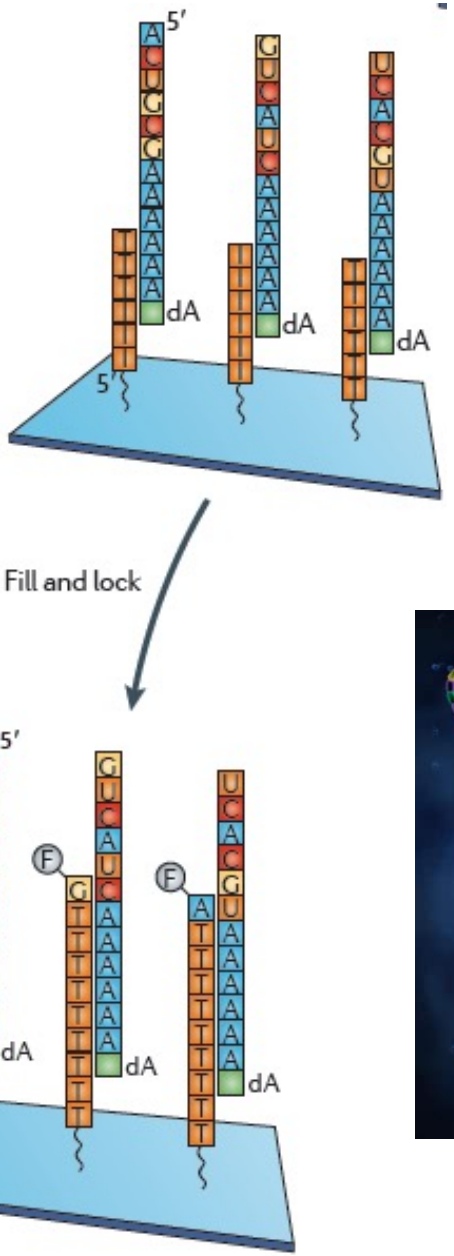
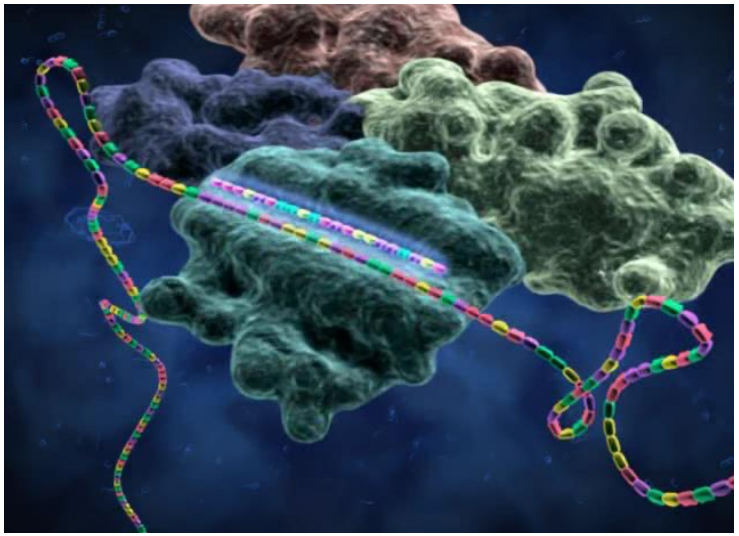
GLOBAL ANALYSES of RNAs and RNPs







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)**

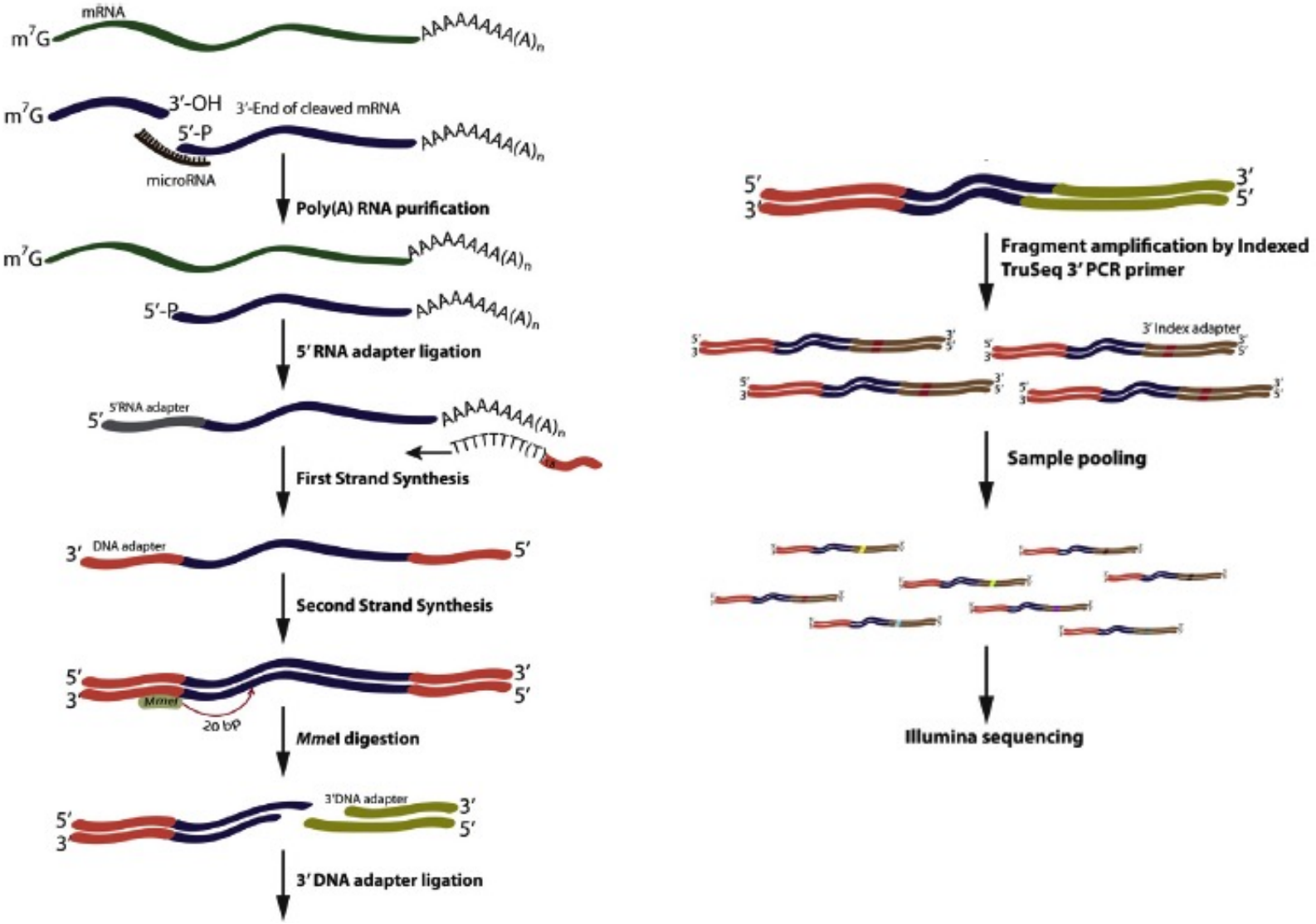
biotinylated oligonucleotides used to enrich for DNA sequences associated with a particular RNA

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

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

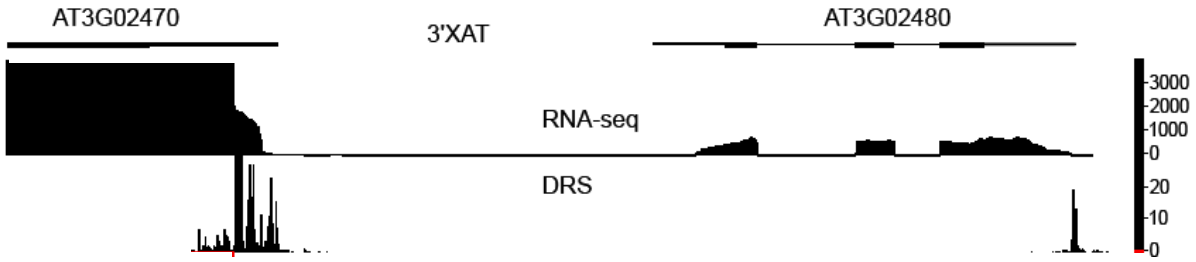
- **HITS-CLIP - High-Throughput Seq CLIP**

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



Zhai et al., Methods, 2014

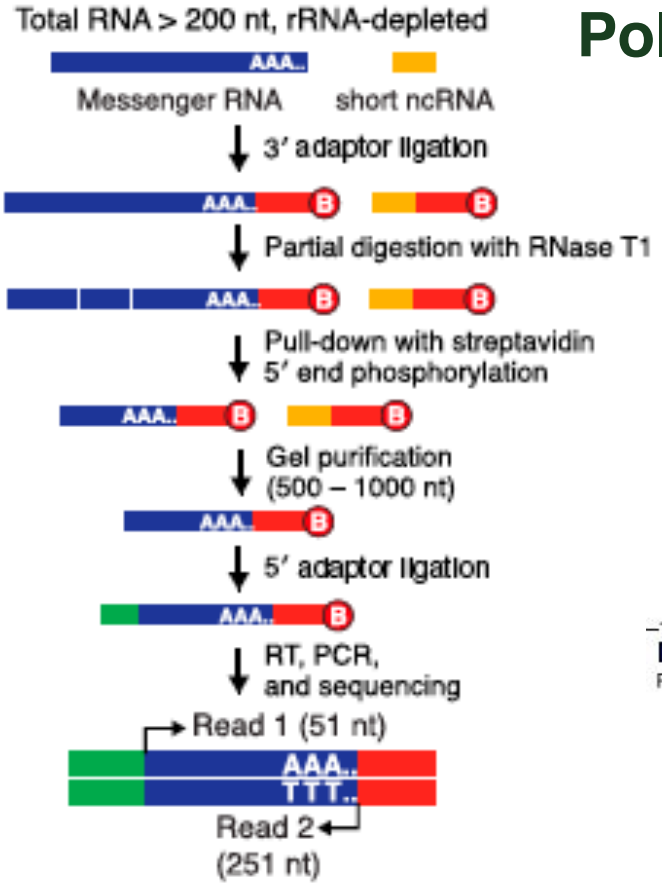
DRS: Direct RNA sequencing of Poly(A) sites

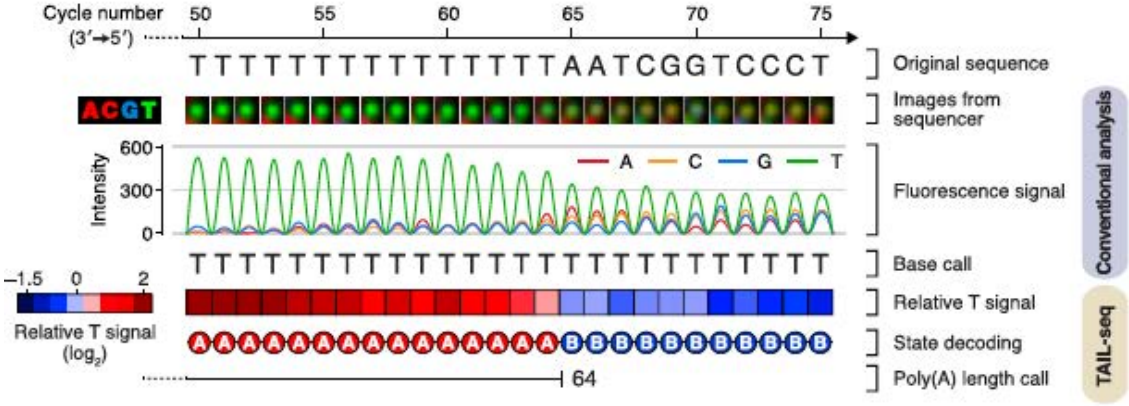


TAIL-seq: RNA 3' end sequencing

Poly(A) tail length and 3' end modifications

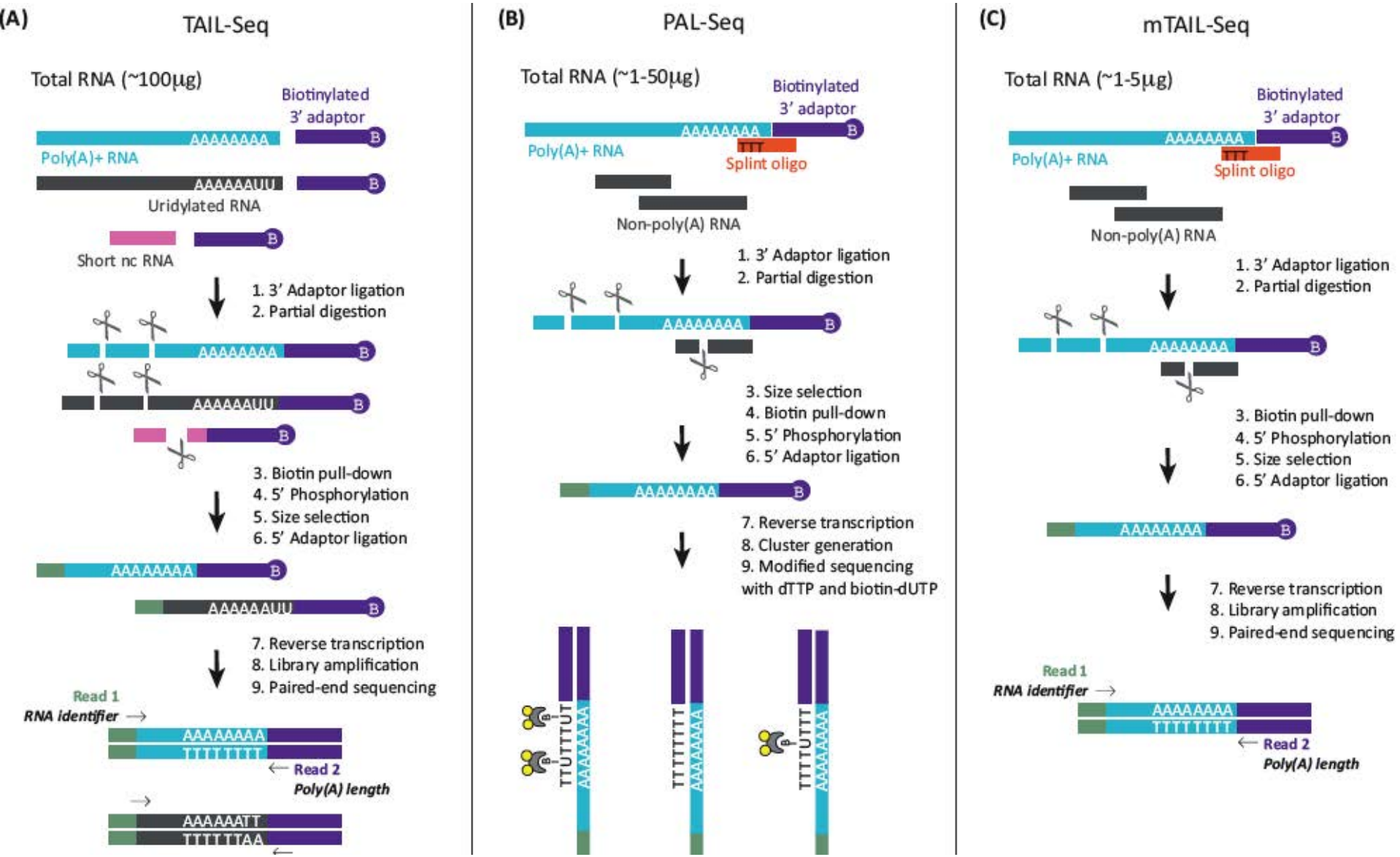
(e.g. U-tailing)





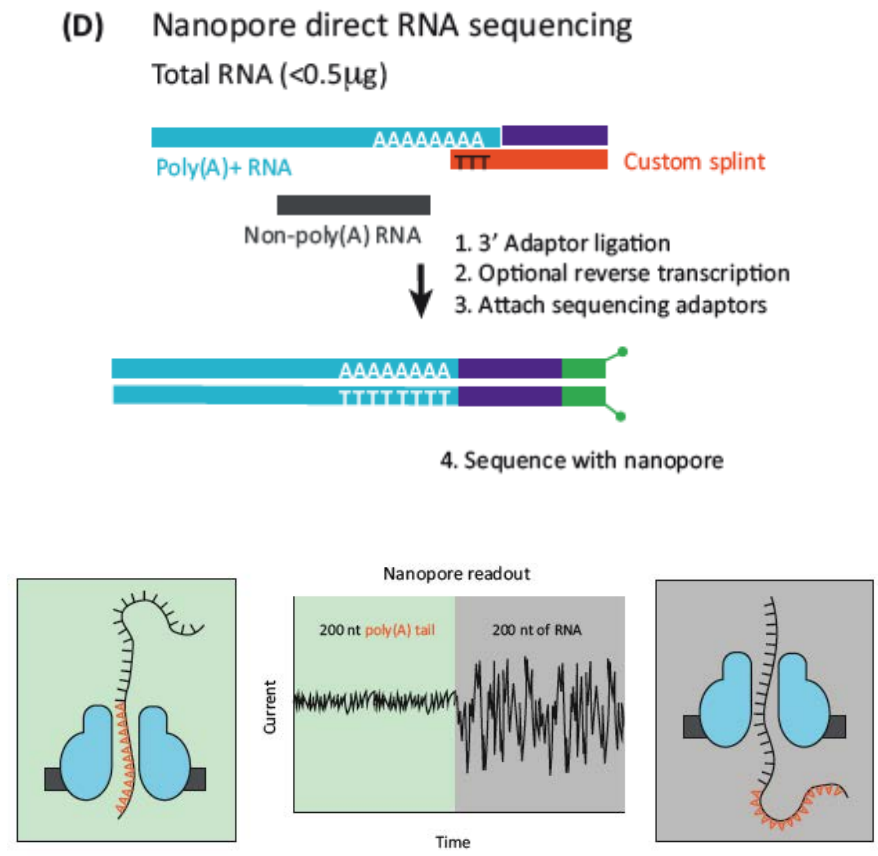
Sherstnev et al., Nat Str Mol Biol, 2012; Chang et al., Mol Cell, 2014

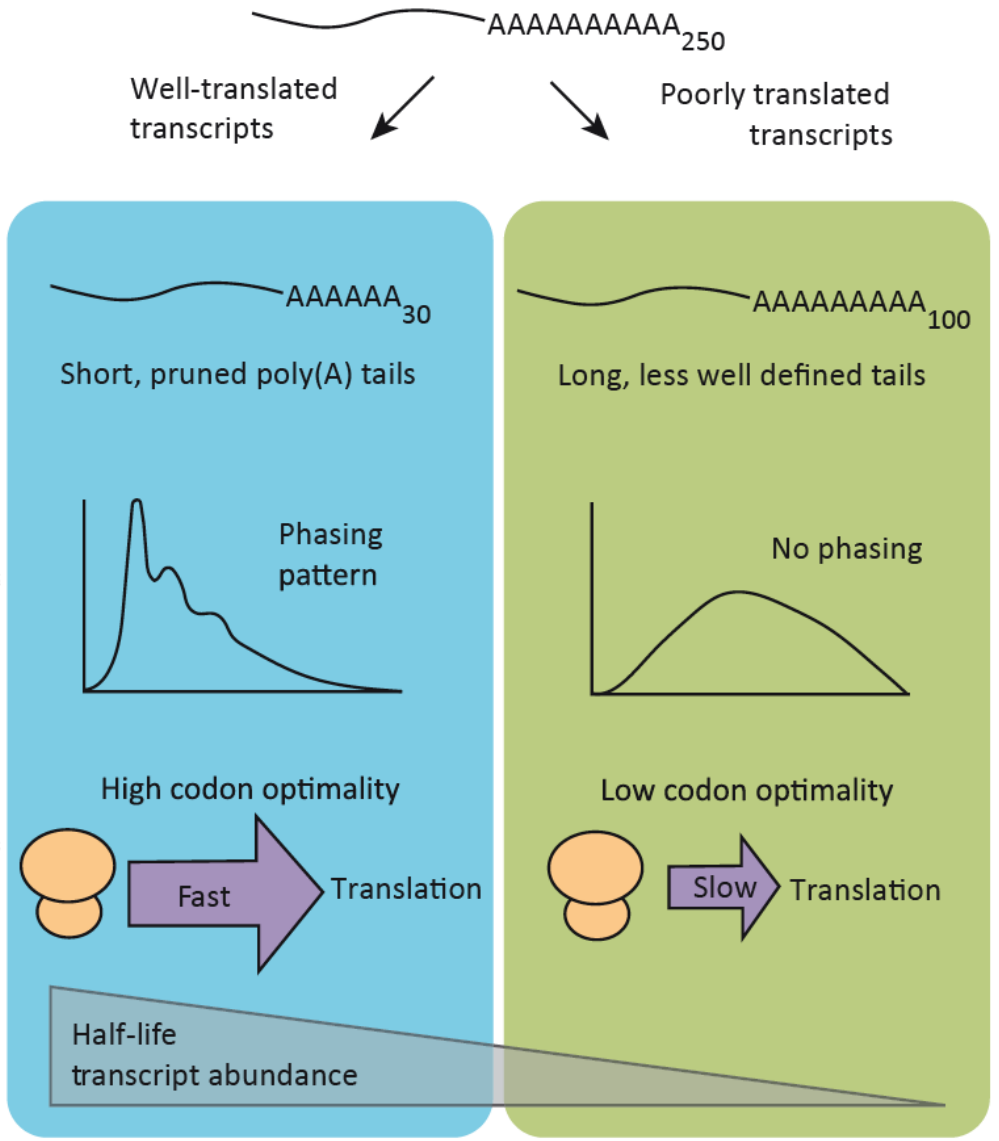
Poly(A) tail analyses



Nicholson and Pasquinelli TiCB 2018

Poly(A) tail analyses Nanopore

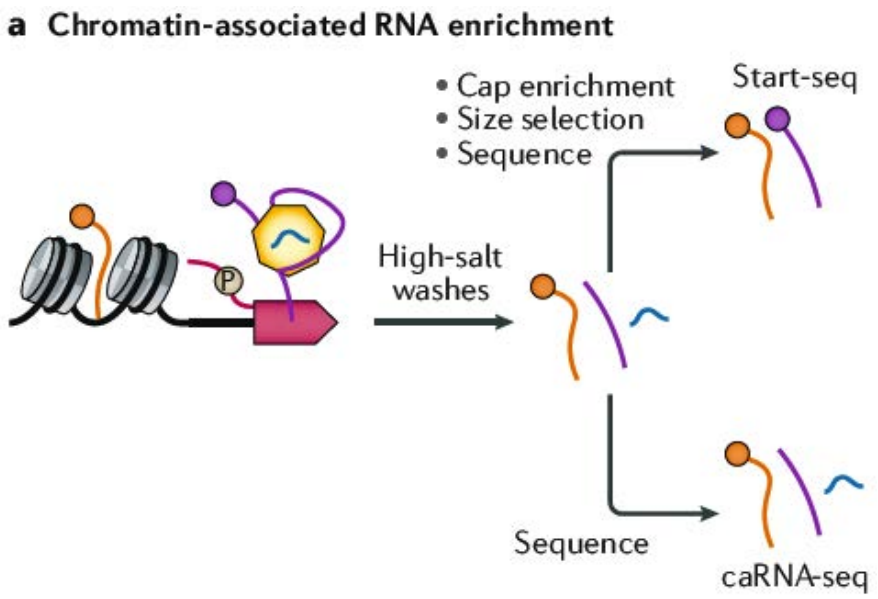


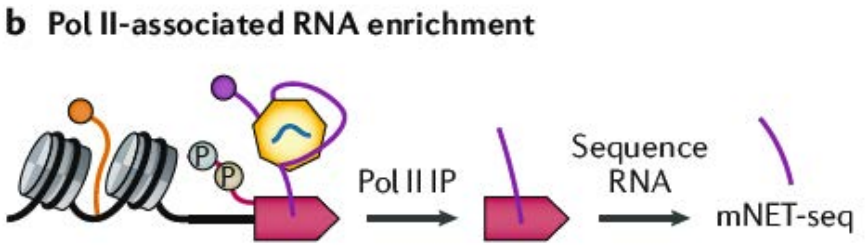


Nicholson and Pasquinelli TiCB 2018

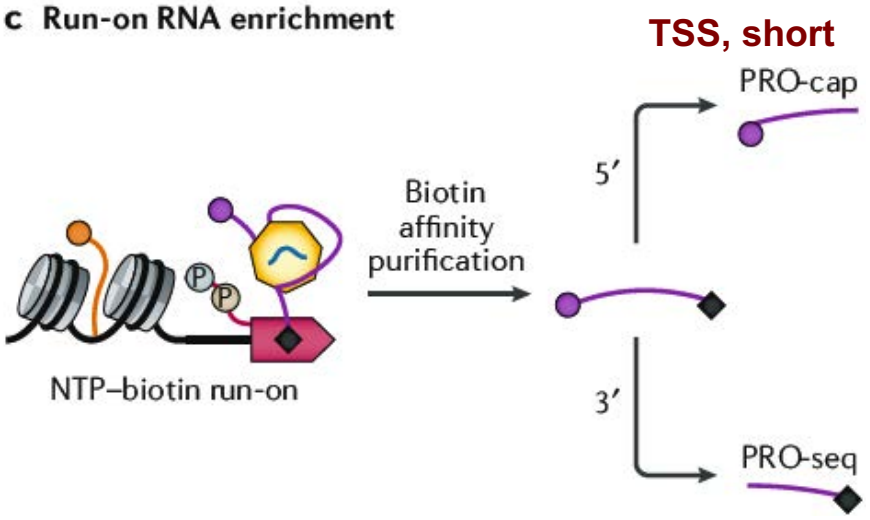
Nascent RNA analyses

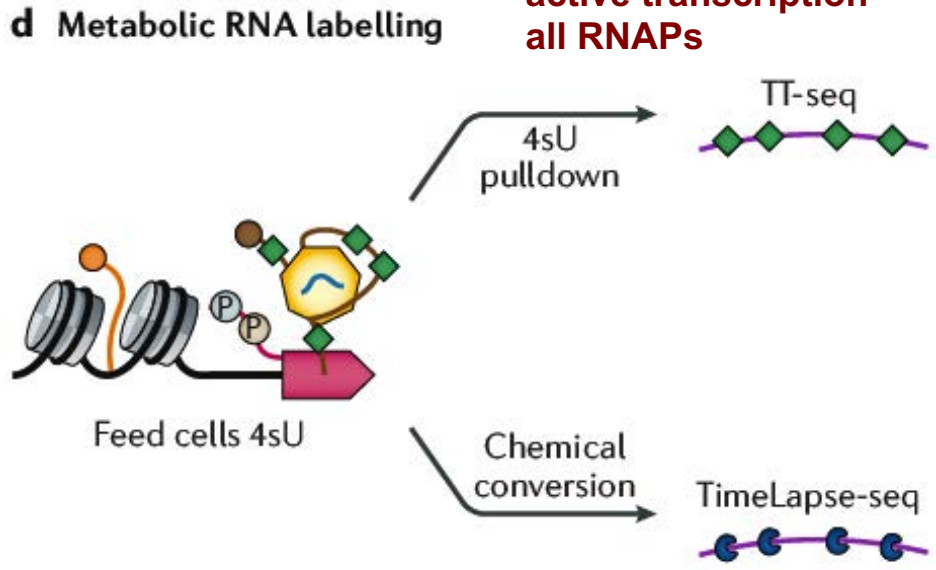
IP-based, formaldehyde crosslink





Purification of transcribed RNAs





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

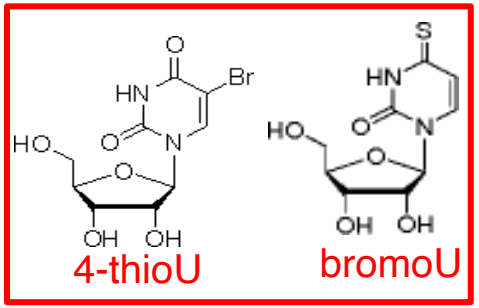
Wissink et al, Nat Rev Genet, 2019

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

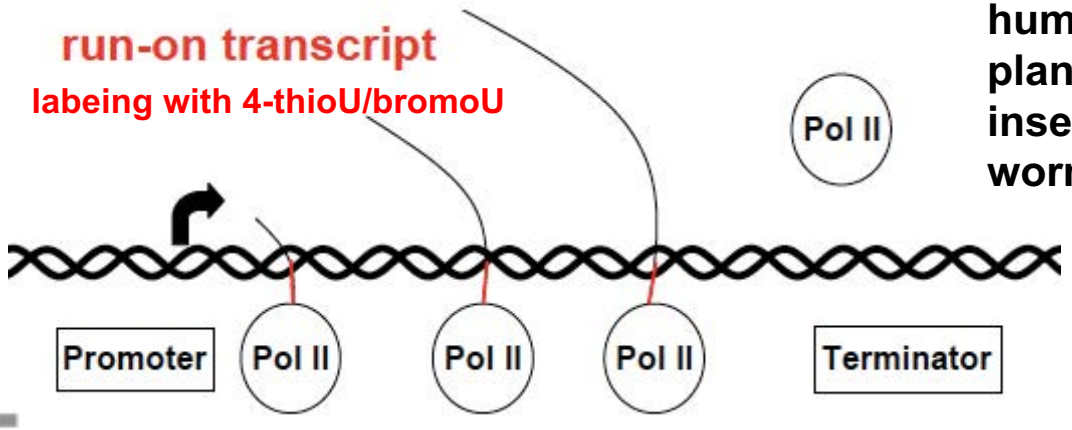
Analysis of Nascent Transcripts- GRO-seq

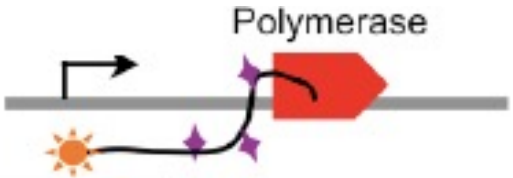


run-on transcript

labeing with 4-thioU/bromoU

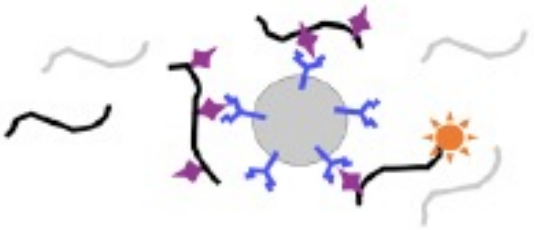
human
plant
insect
worm





Isolate and hyrdrolyze RNA

Bead Binding



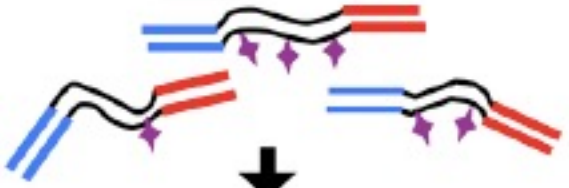
Wash / Elute

Cap removal

5' adapter ligation

3' adapter ligation

Reverse Transcription



Amplification

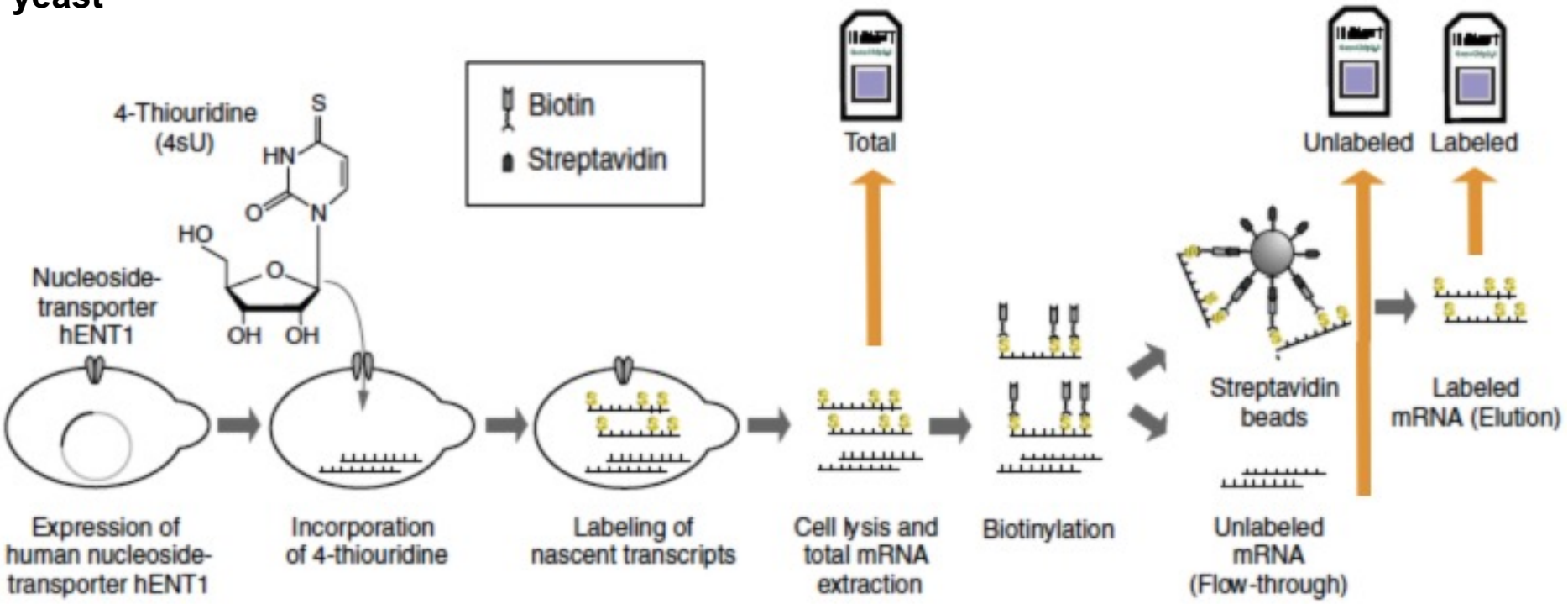
PAGE Purifcation

5' end MPSS
Illumina 1G Genome Analyzer

Core et al., Science, 2010

Analysis of Nascent Transcripts

yeast



Expression of hENT1 nucleoside transporter enables uptake of UTP derivatives

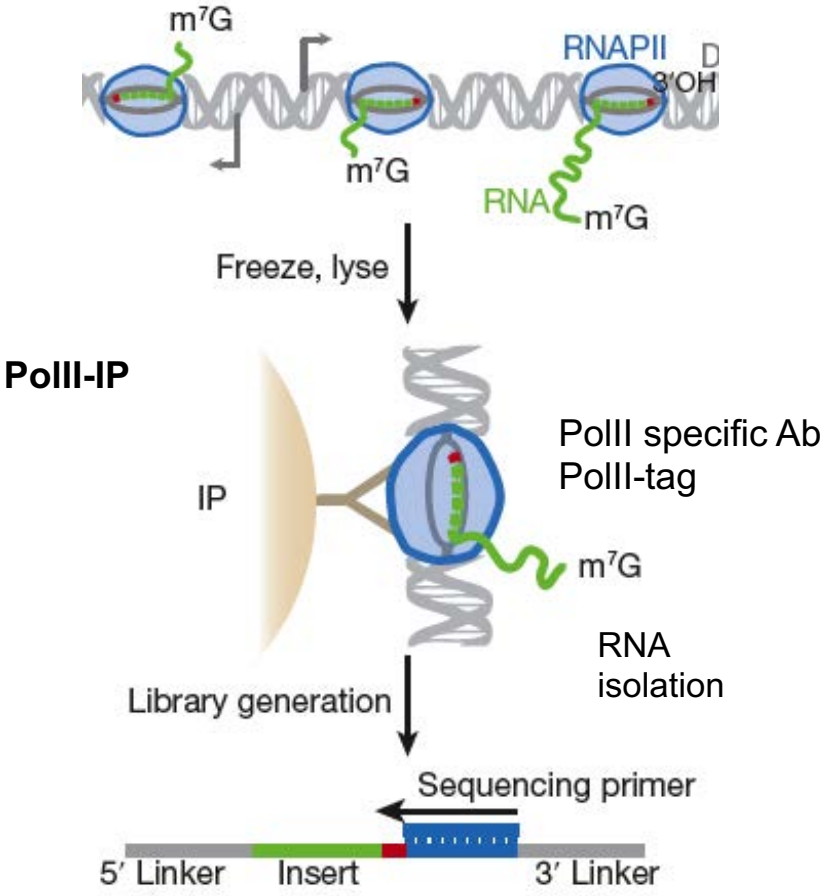
Non-perturbing RNA labeling in yeast
Allows dynamic transcriptome analysis: sythesis and decay rates and the study of nascent transcripts

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

Analysis of Nascent Transcripts

NET-seq

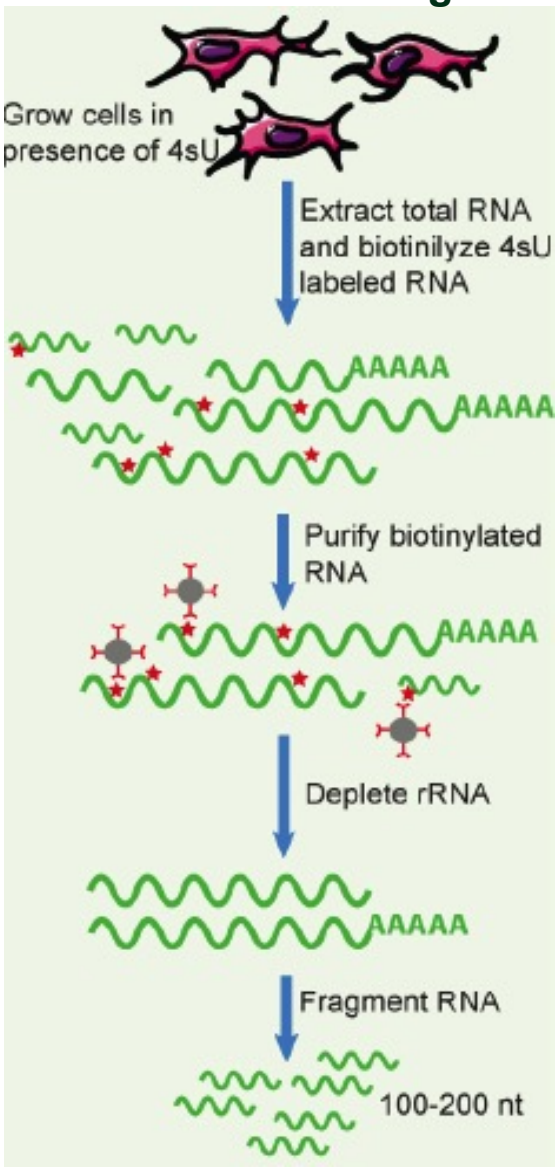
I. Isolation of PolII-bound RNAs



Churchman and Weissman, Nature, 2011

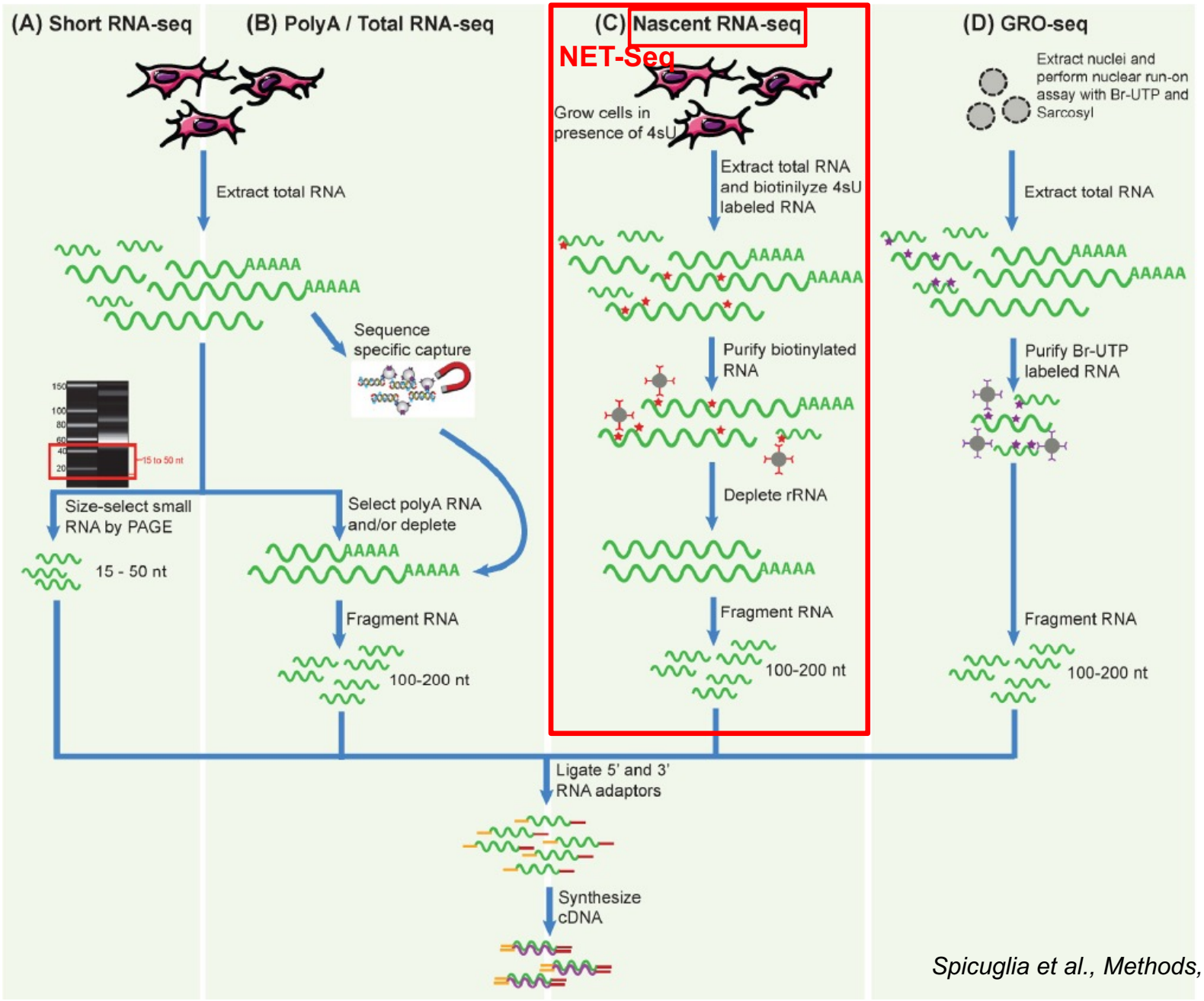
GRO-seq

II. Nascent RNA labeling with 4sU



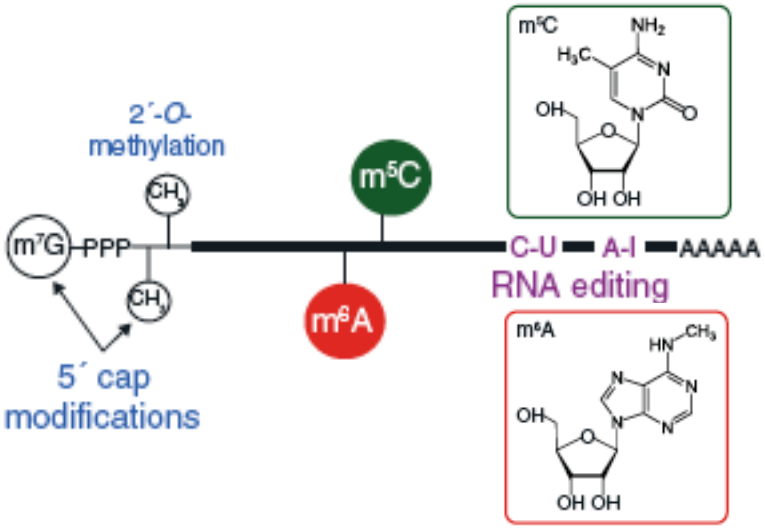
Spicuglia et al., Methods, 2013

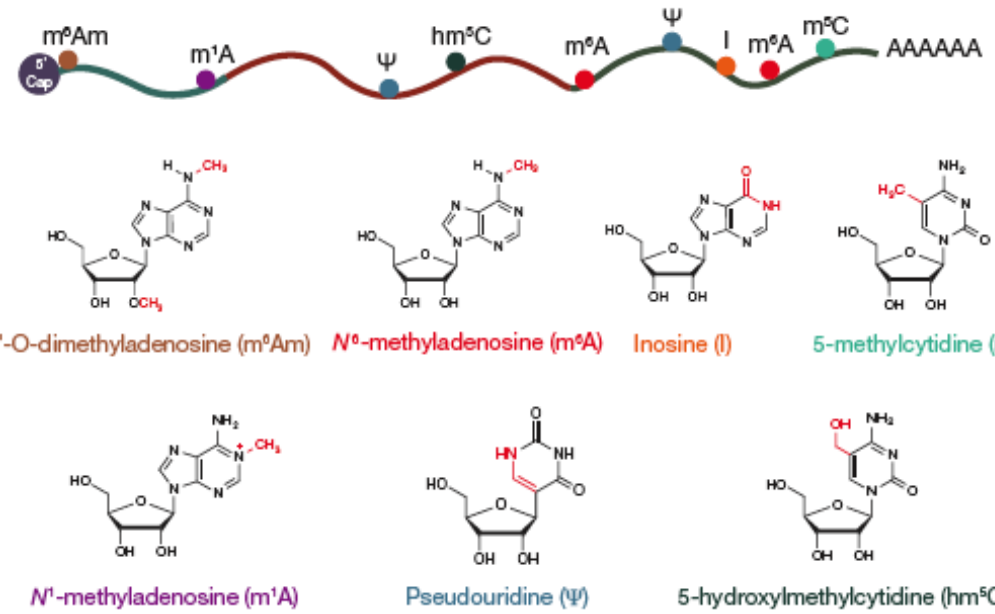
Comparison of different RNA-Seq approaches



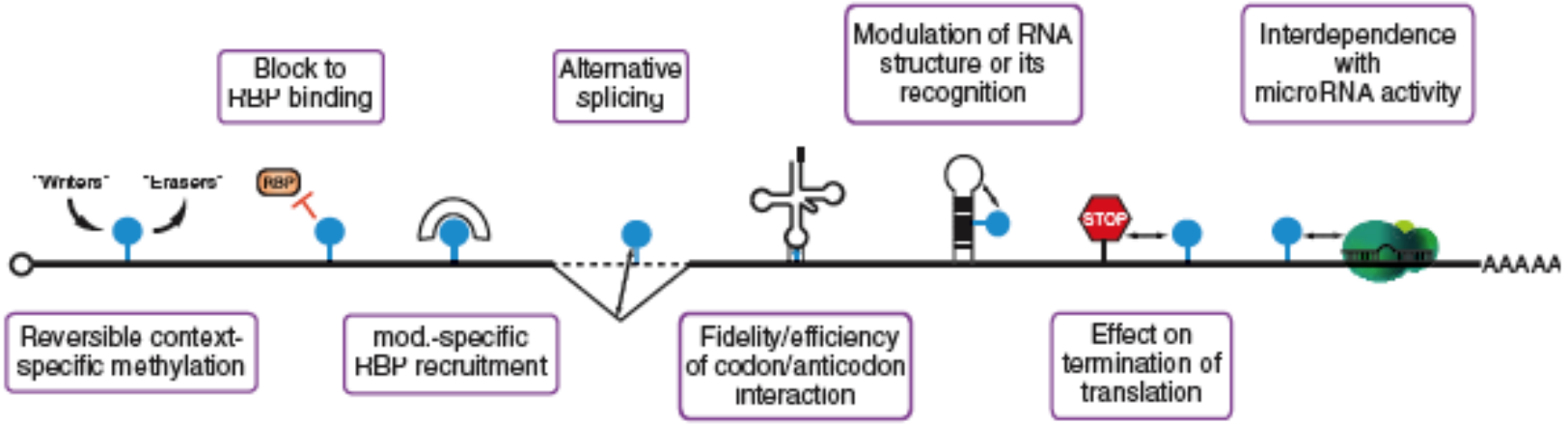
Spicuglia et al., Methods, 2013

RNA MODIFICATIONS





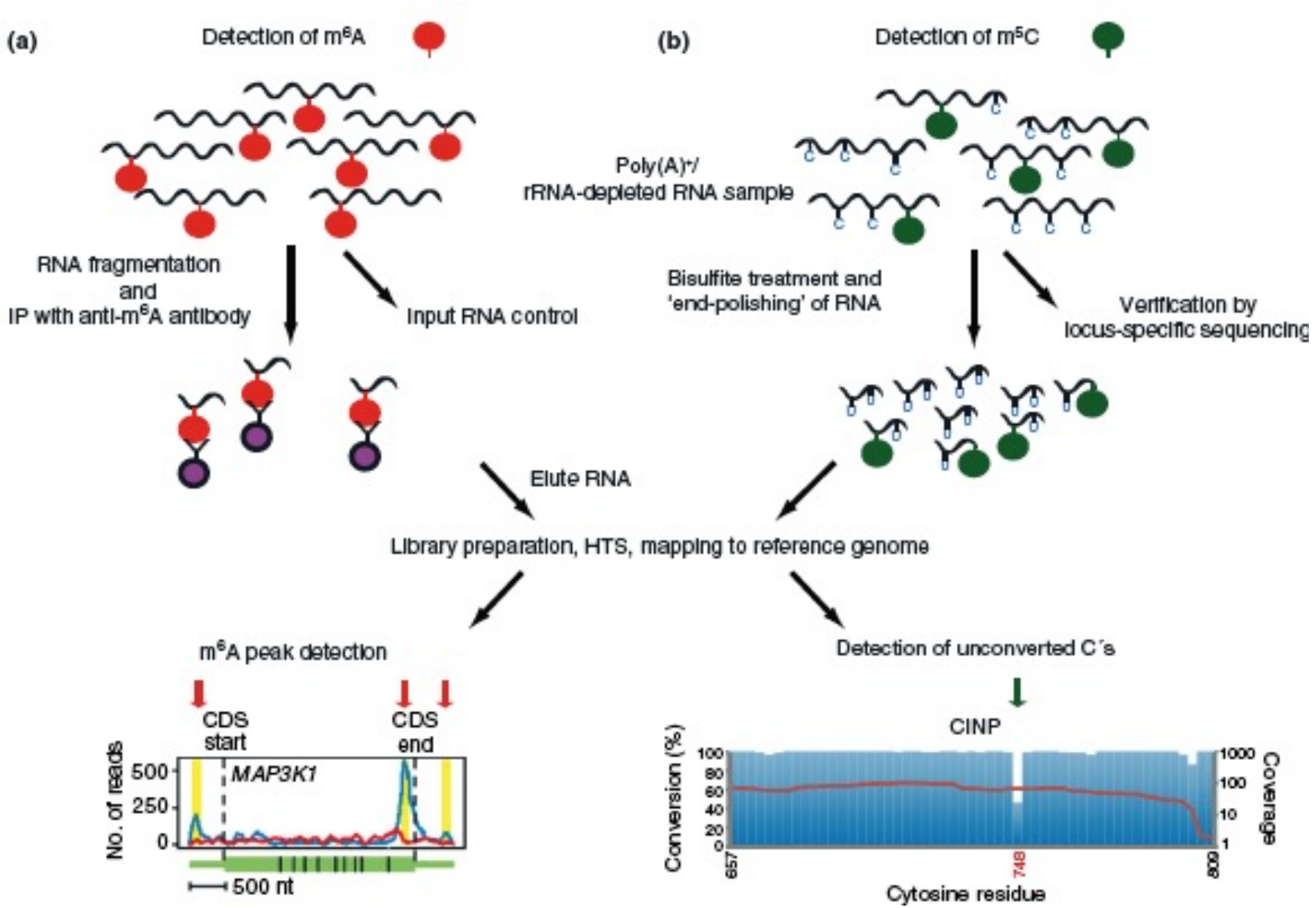
FUNCTIONS



mRNA splicing, export, localisation, decay, translation, innate immunity

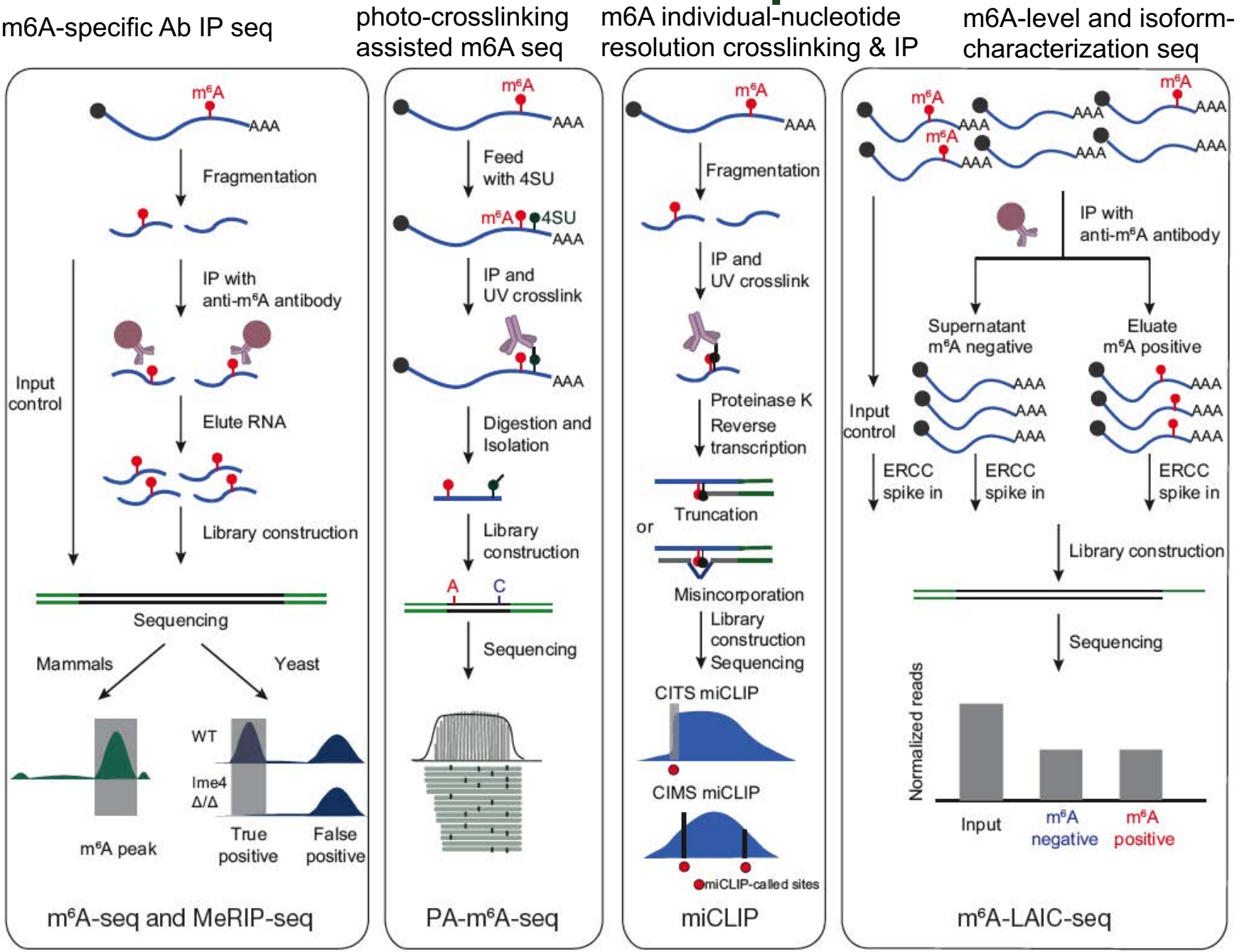
Sibbrit et al, WIREsRNA 2013

RNA MODIFICATION



Sibbrrit et al, WIREsRNA 2013

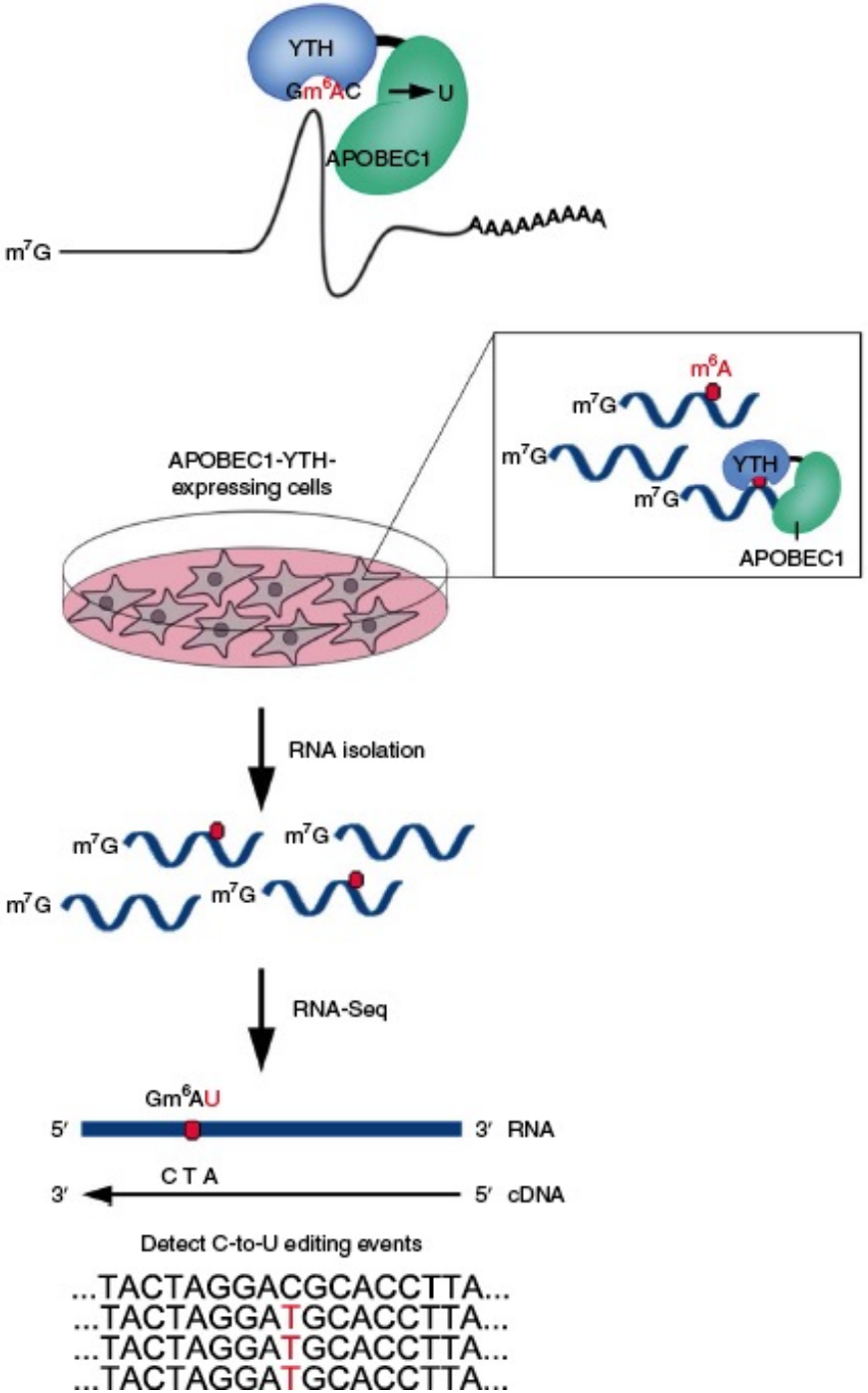
m⁶A RNA-seq



Li at al, Nat Methods, 2017

antibody-free m6A-seq DART-seq

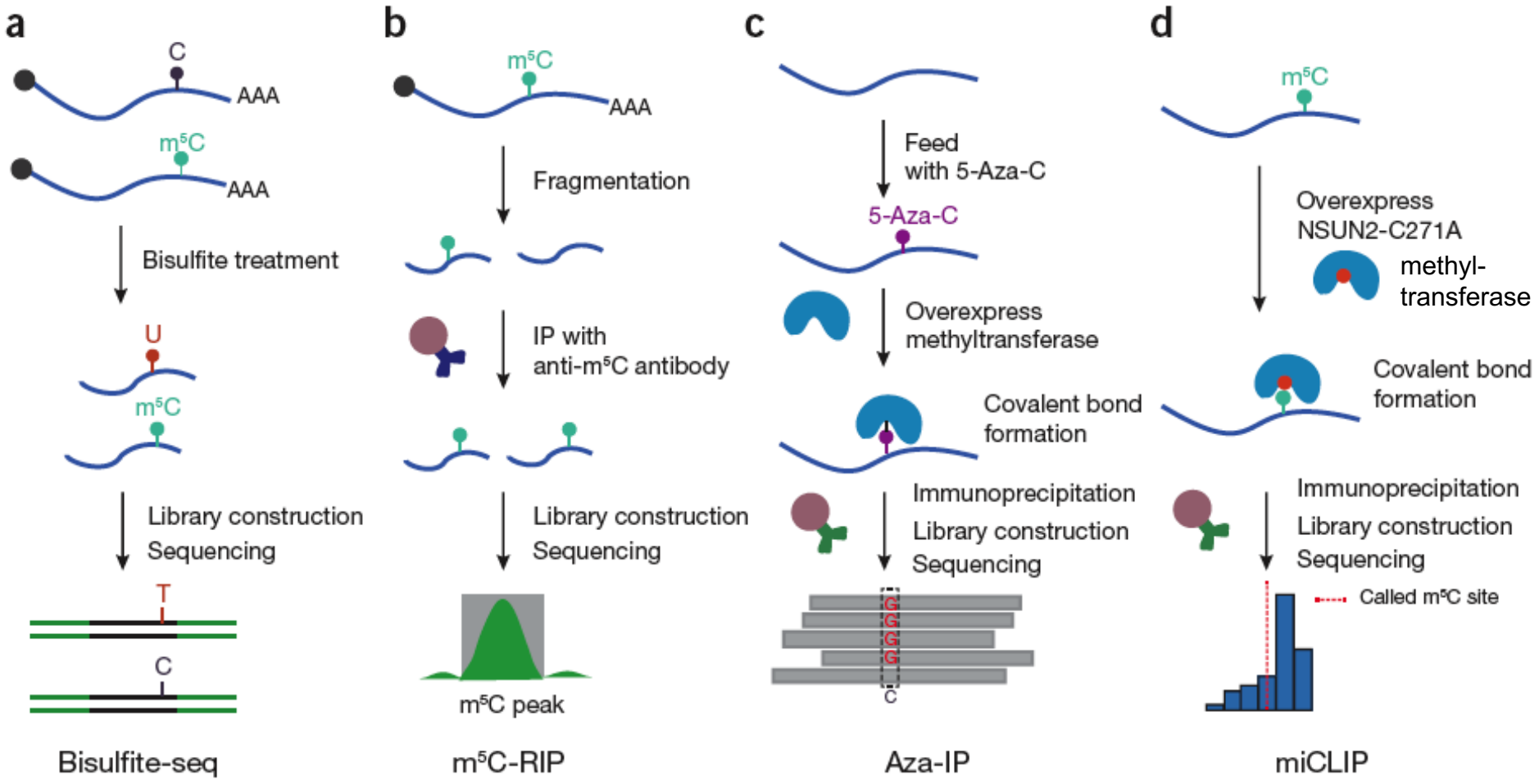
deamination adjacent to RNA modification targets



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

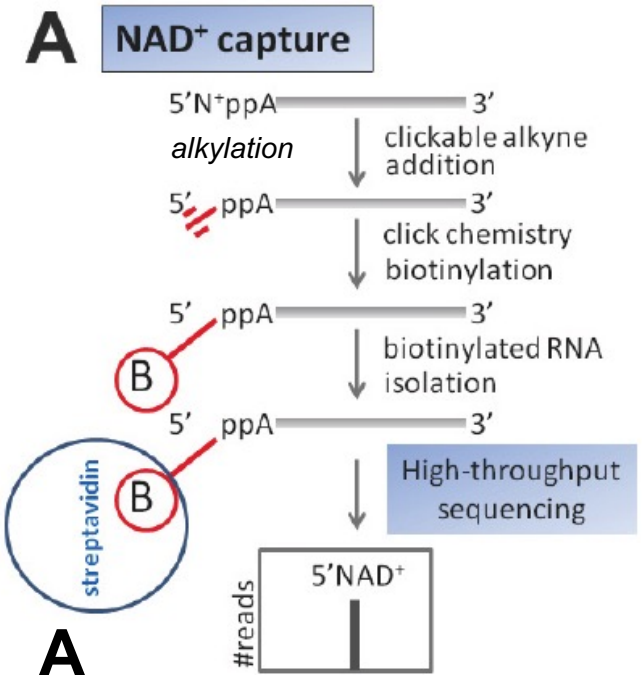
Meyer, Nature Methods 2019

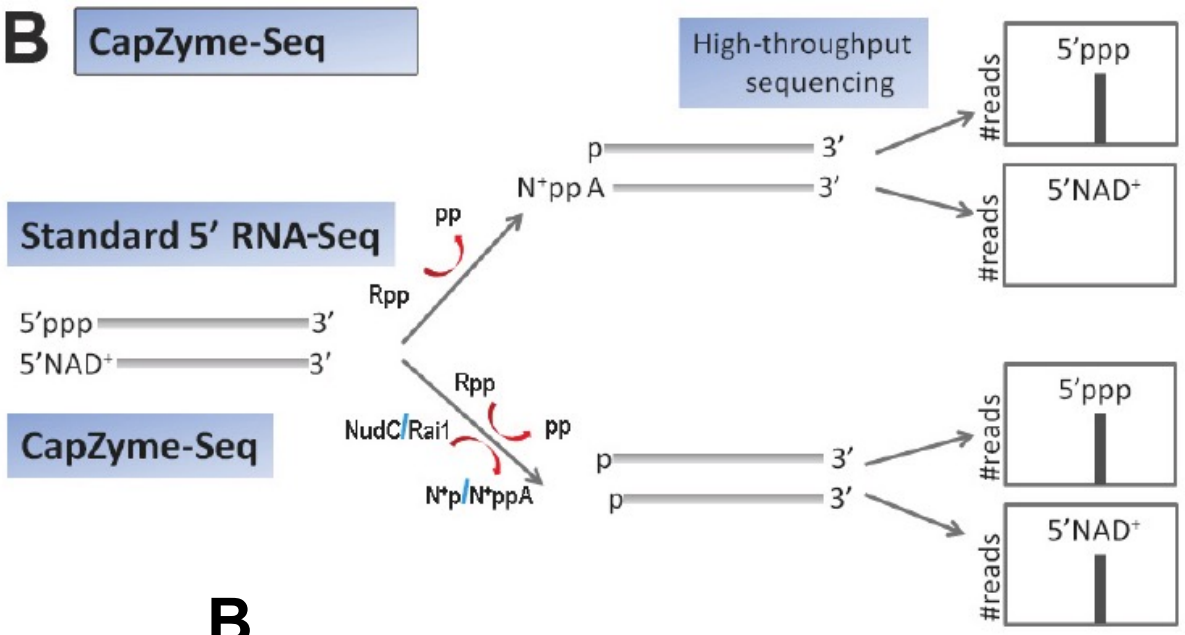
m^{5}C RNA-seq

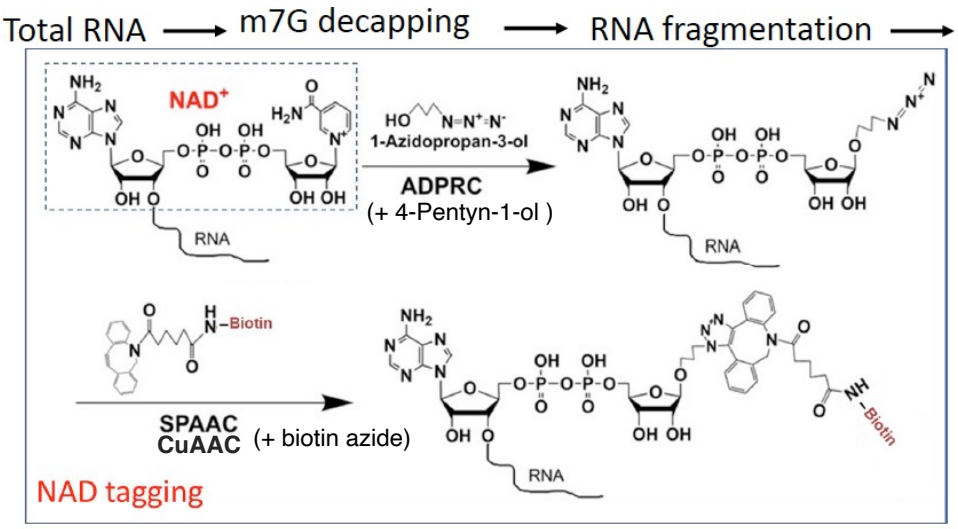


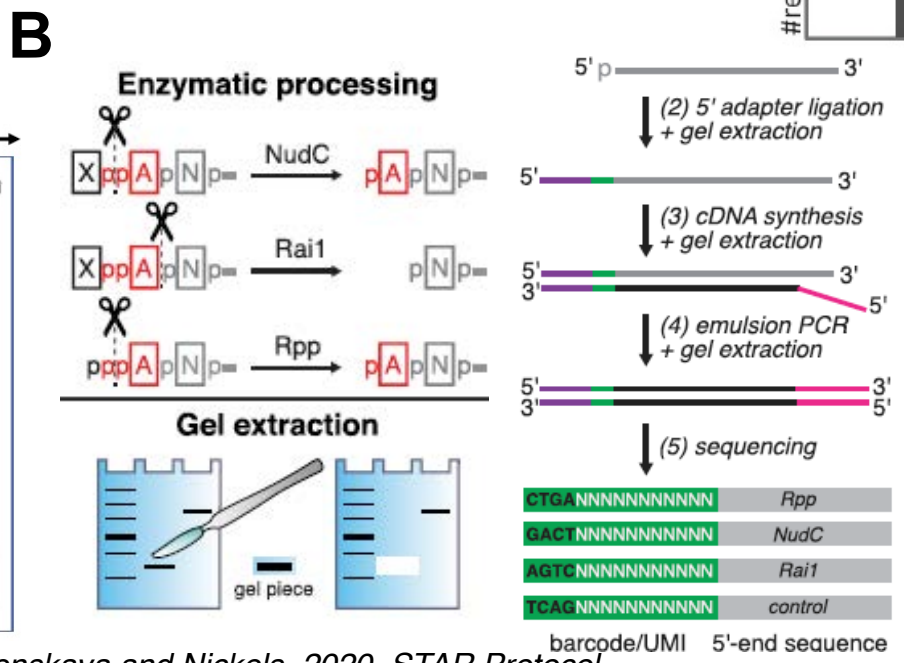
Li at al, Nat Methods, 2017

Identification of NAD+ capped RNAs









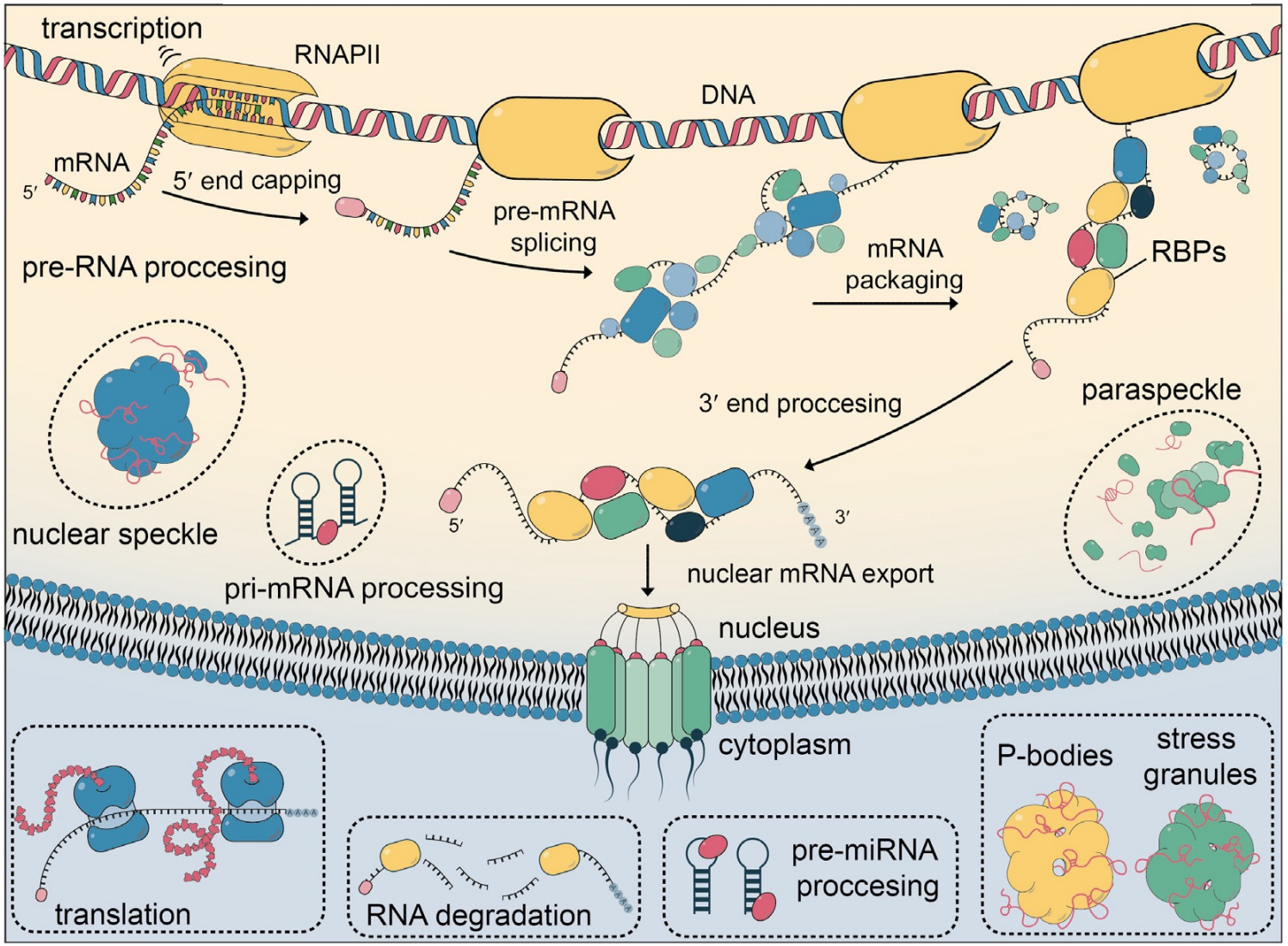
Adres Jasche (2016); Yiji Xia (2018-2022)

Vvedenskaya and Nickels, 2020, STAR Protocol

INTERACTIONS:

RNA-proteins
RNA-DNA
RNA-RNA
RNA structure

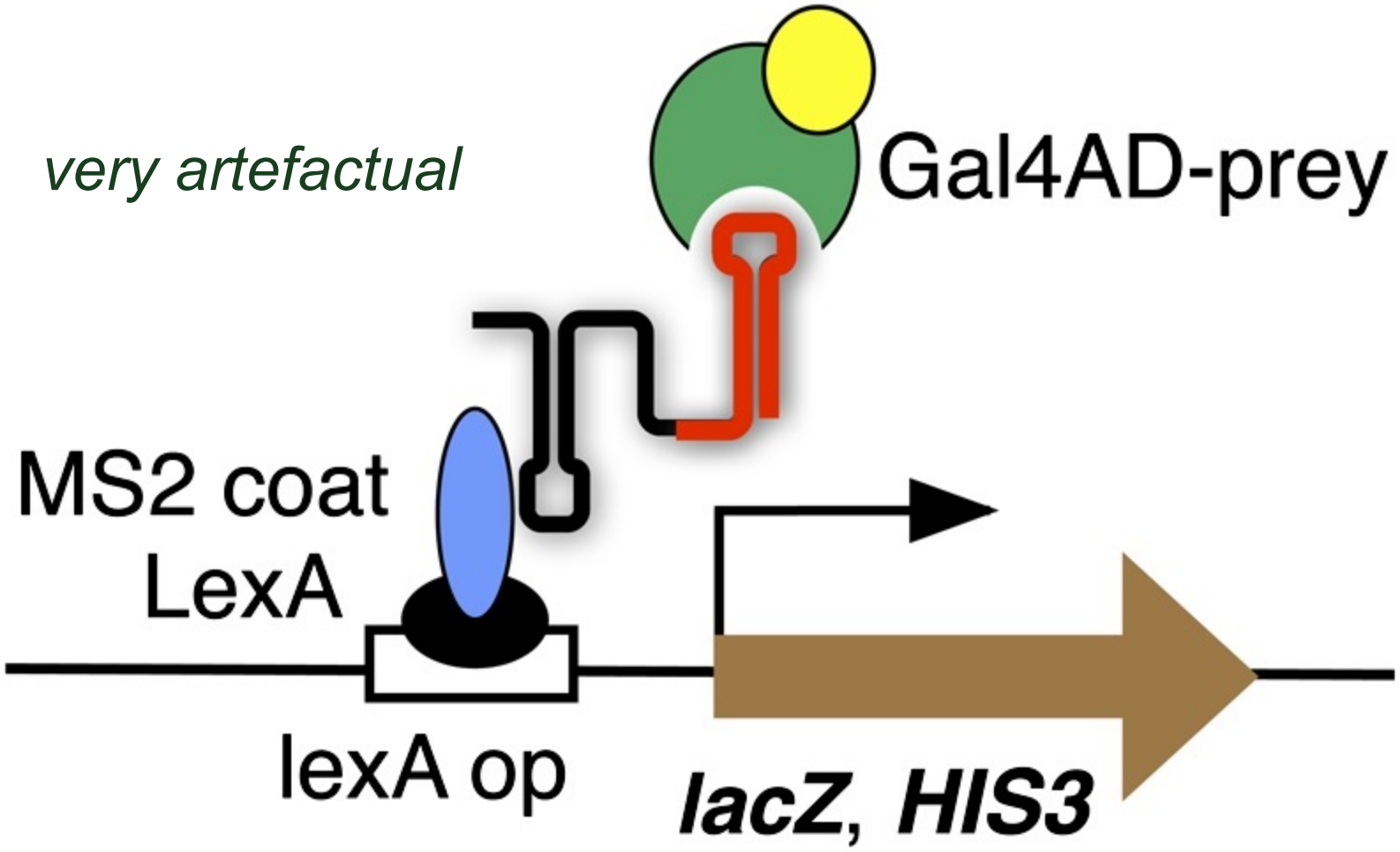
RBP - RNA binding proteins



Kilchert et al., WiRES RNA, 2020

- **facilitate each step of RNA biogenesis**
- **participate in cellular procesess- transcription, export, translation, RNA decay**
- **form RNPs and subcellular granules and organelles**

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).

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

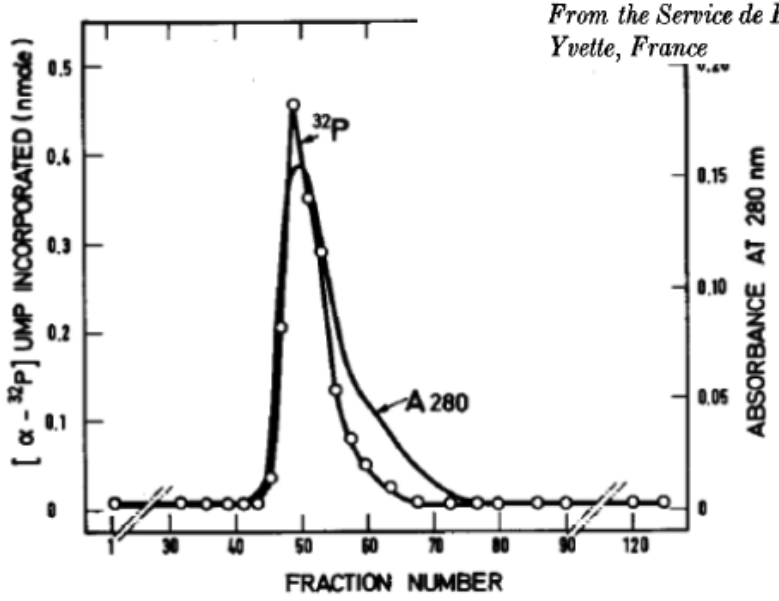


FIG. 1. DEAE-cellulose column chromatography. Fraction 3 (15 ml, $A_{280\ nm}$ 0.8) was applied to a column (5 cm^2 × 16 cm) of DEAE-cellulose and eluted as described in the text. Fractions of 3 ml were collected and assayed for RNA polymerase activity on 10-μl aliquots for 10 min under standard conditions.

TABLE I

Summary of RNA polymerase A purification

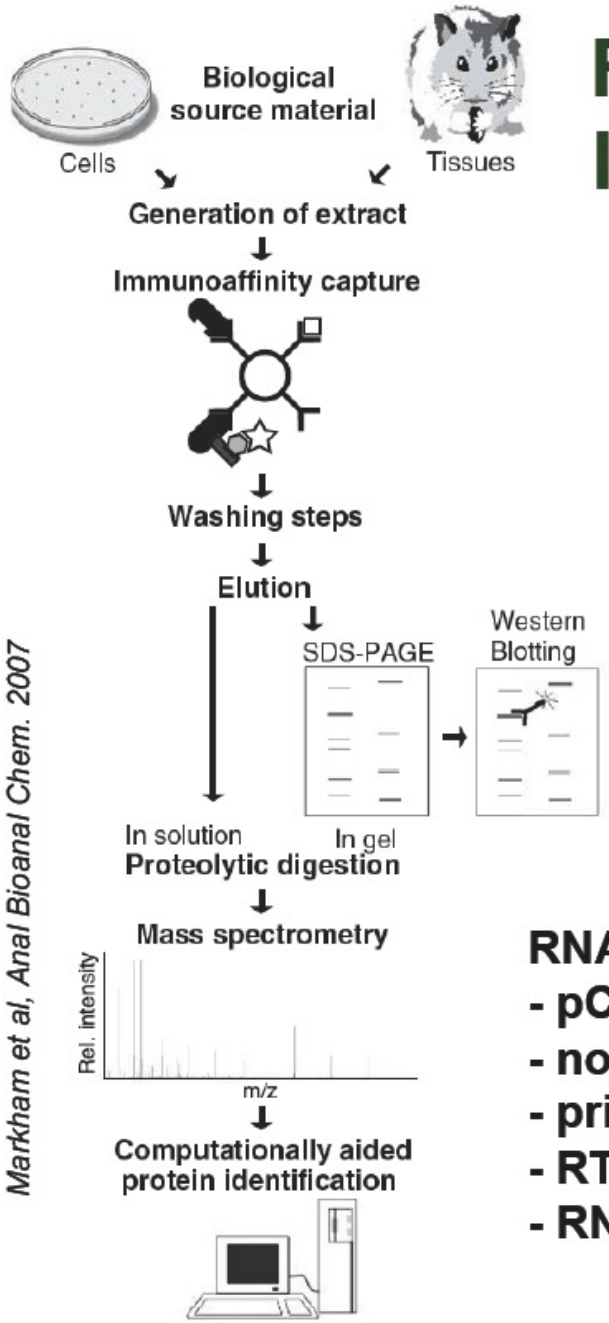
Values are given for 300 g of yeast cells.

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.

RNP IMMUNOPRECIPITATION
IP, co-IP

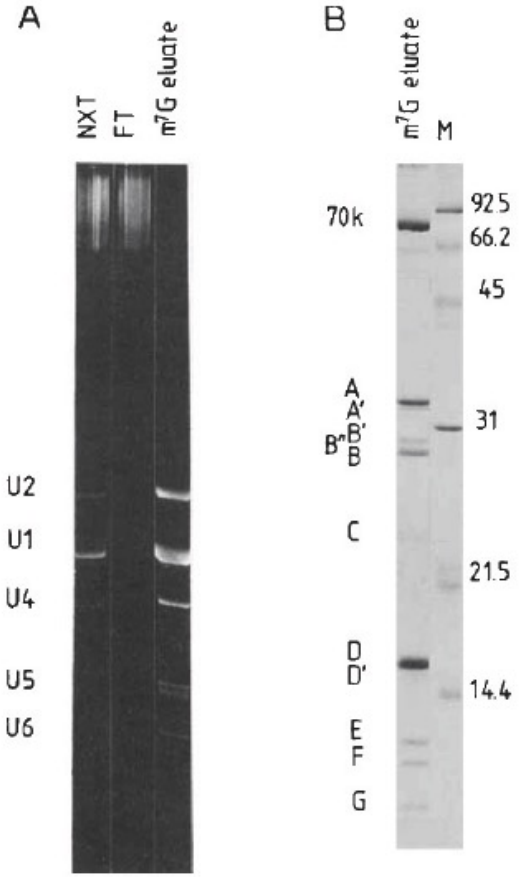
With specific antibodies or using tagged proteins



Markham et al, Anal Bioanal Chem. 2007

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

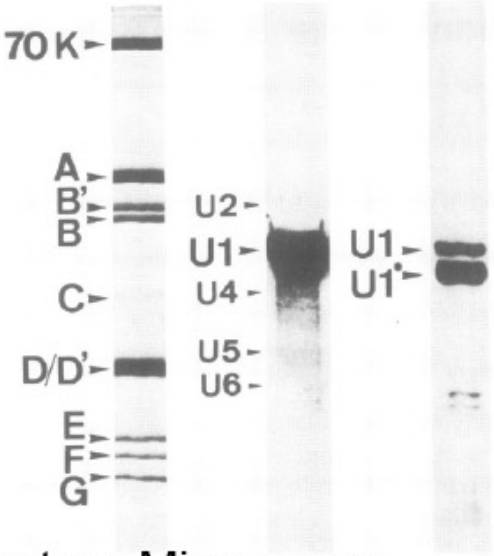
U snRNPs with anti-TMG cap antibody



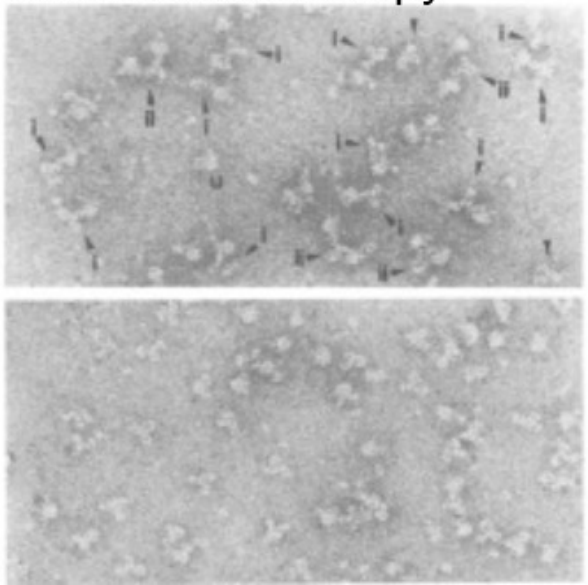
Bochnig et al, Eur. J Biochem. 1987
(Luhrmann's lab)

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

Immunoaffinity +ion exchange



Electron Microscopy



IP of snRNPs with anti-TMG cap Ab

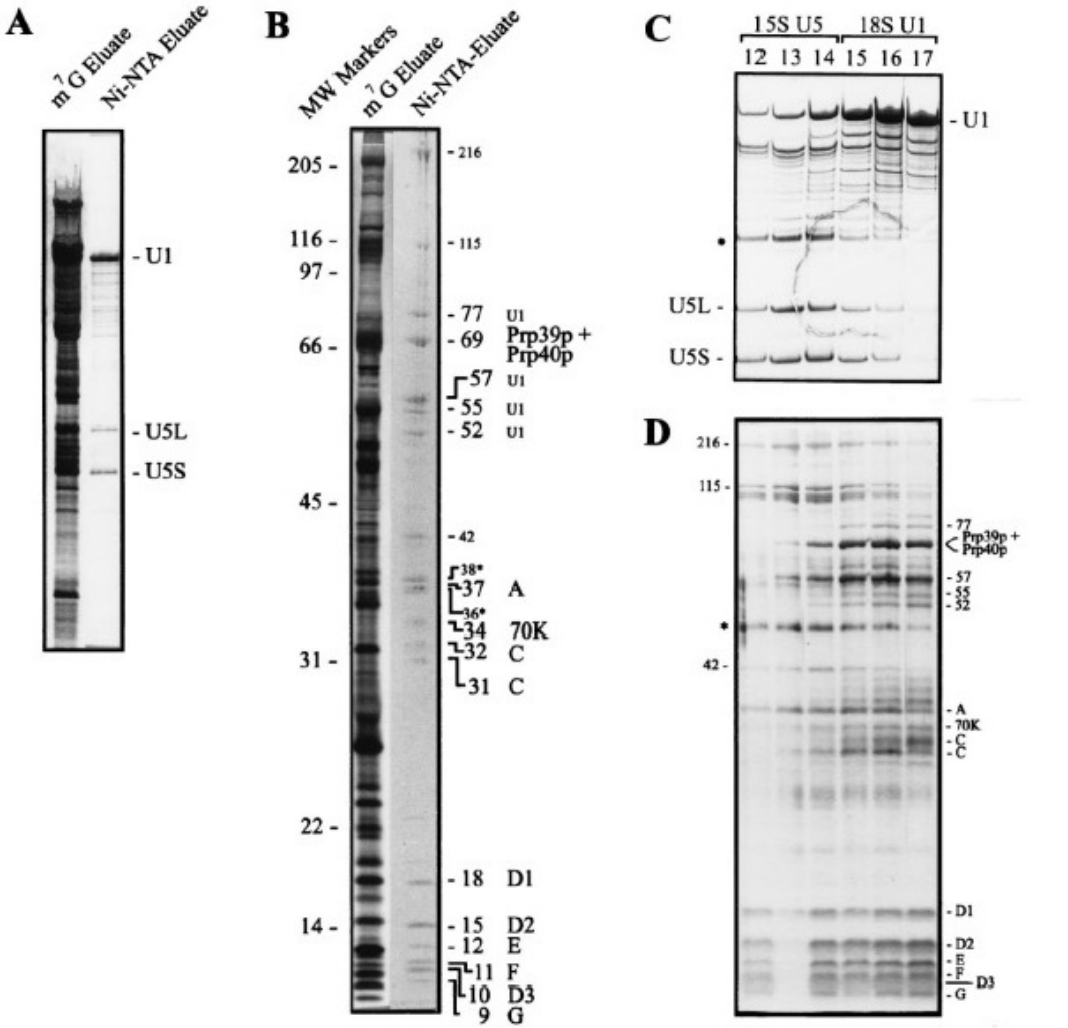
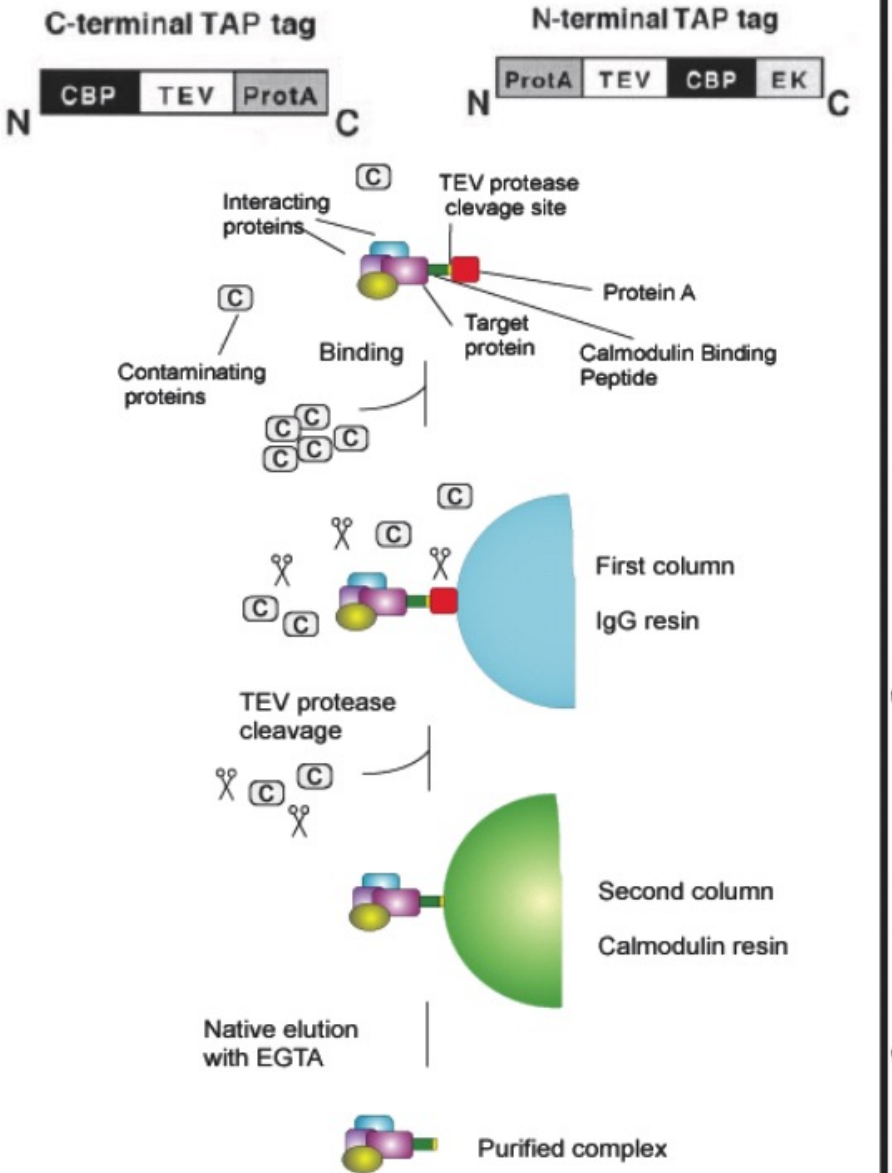
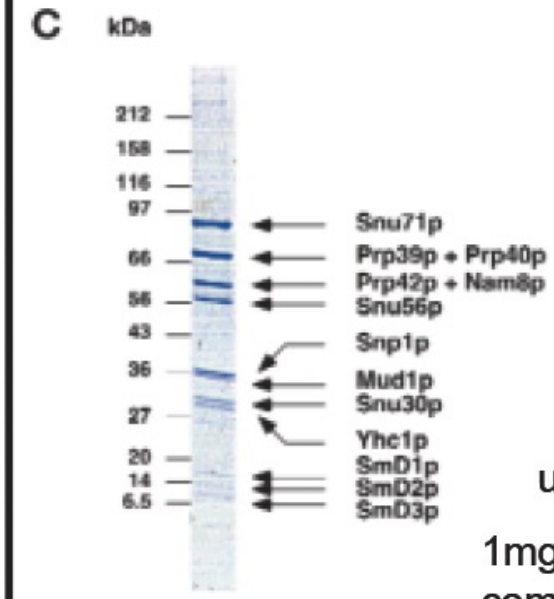
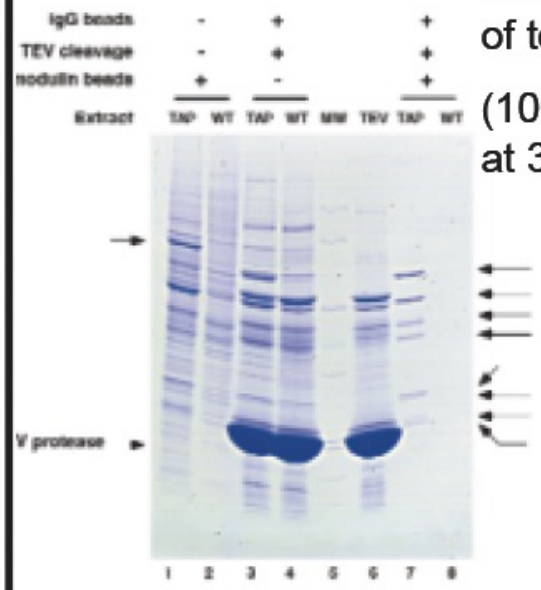


FIG. 1. Purification of U1 snRNPs from *S. cerevisiae*. (*A*) Silver staining of snRNAs eluted from anti-m_3G-cap (m^7G eluate) and Ni-NTA affinity

TANDEM AFFINITY PURIFICATION (TAP)







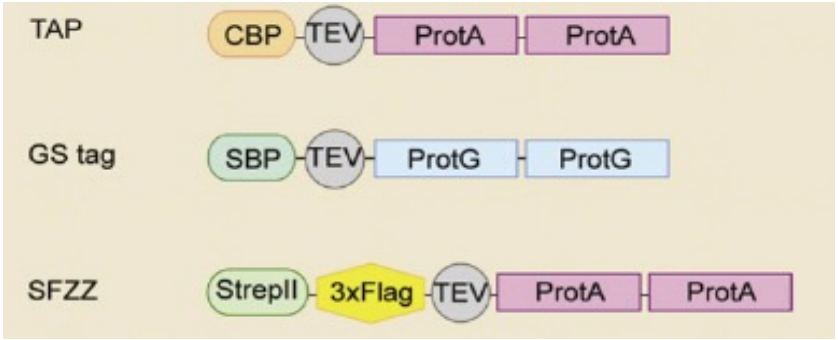
usual yield:

1mg of protein complex from 300mg of total protein

(100 000 X purification at 30% efficiency)

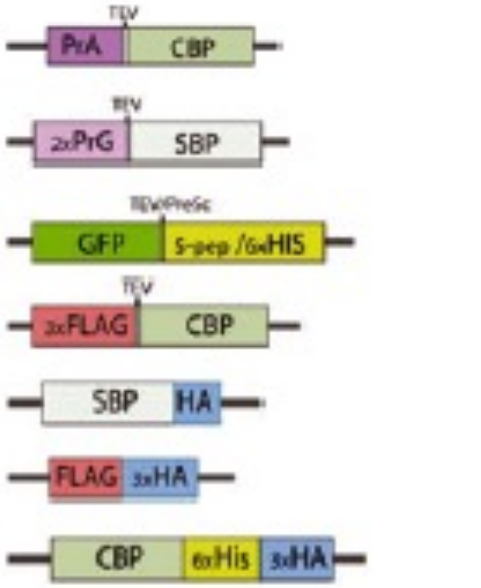
MODIFIED TAP tags

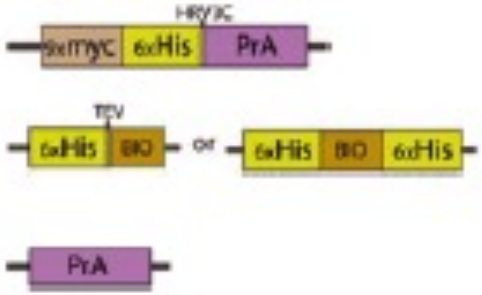
mammalian cells



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

Tandem

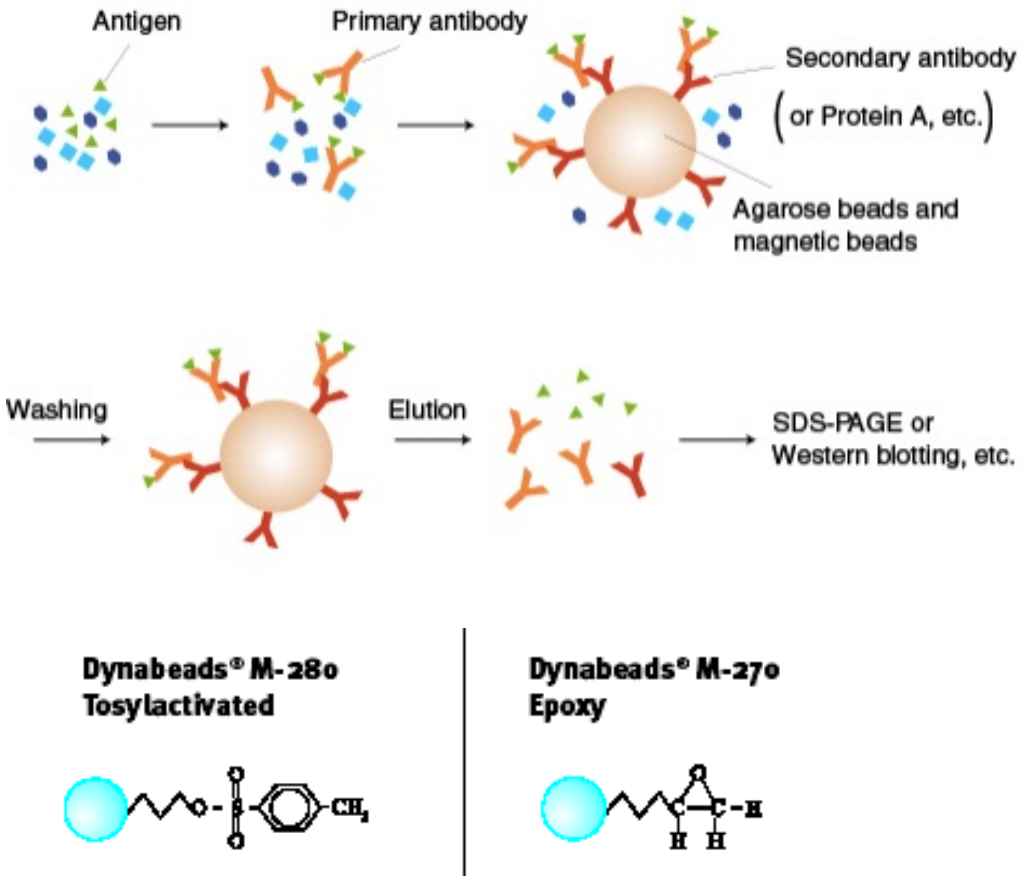


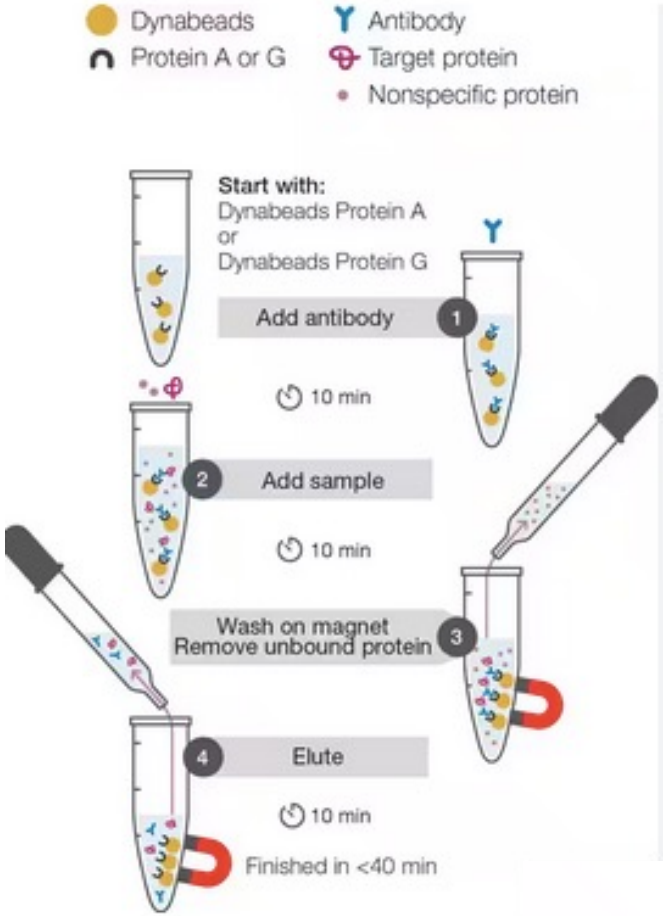


Single-step

MAGNETIC versus AGAROSE beads

- Agarose beads - very low background and high binding capacity IP (centrifugation)
- Magnetic Agarose beads - magnetic separation, high binding capacity IP, fast, easy
- Magnetic Particles M-270 - IP of very large proteins/complexes, fast





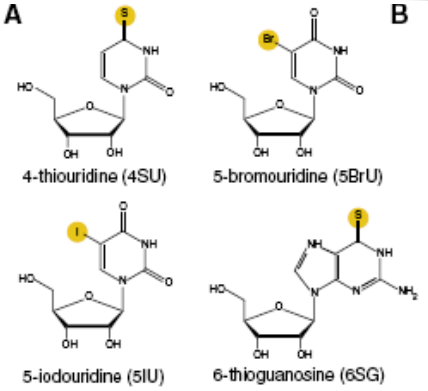
Diameter 2.8 um volume 40 000 smaller than agarose/sepharose

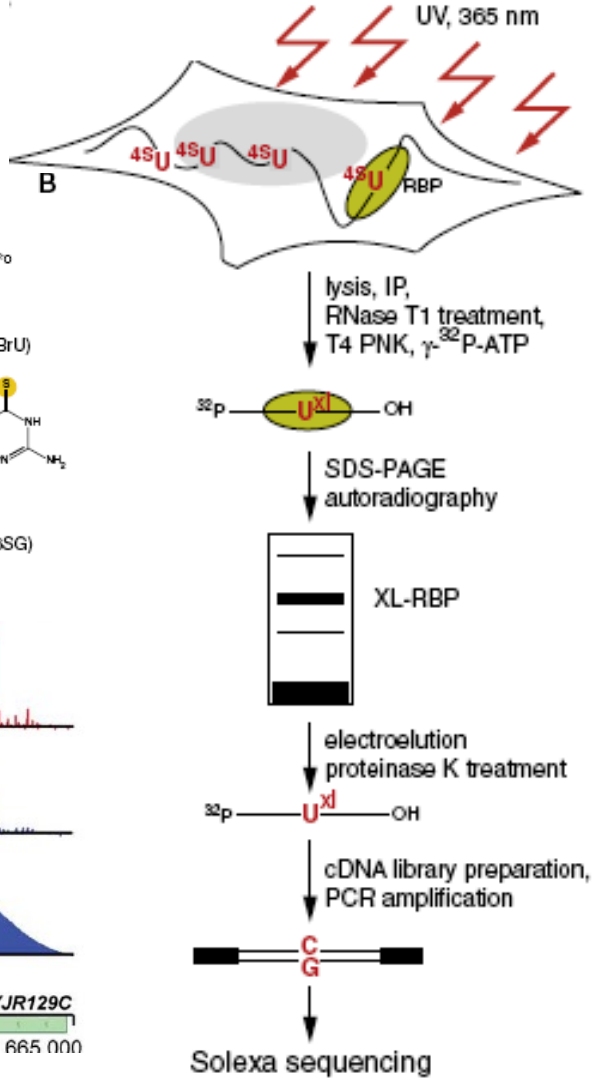
PAR-CLIP

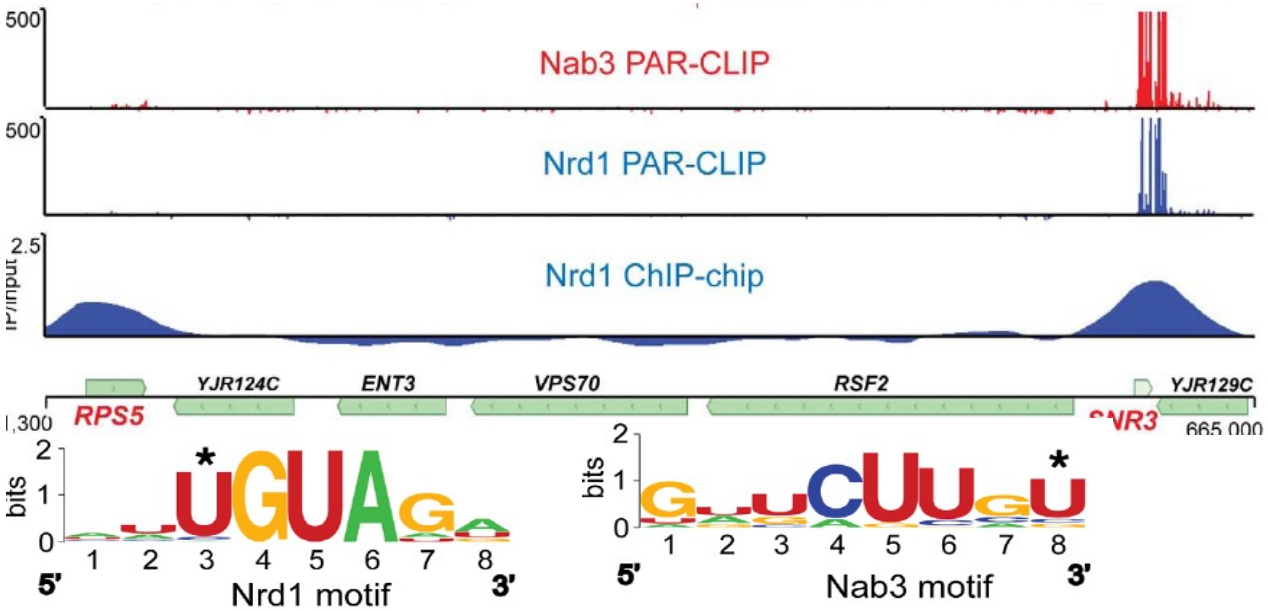
PhotoActivatable ribonucleoside–enhanced CrossLinking and ImmunoPrecipitation

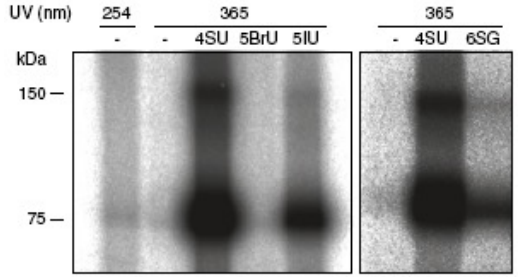
HITS-CLIP:

High-Throughput Seq CLIP







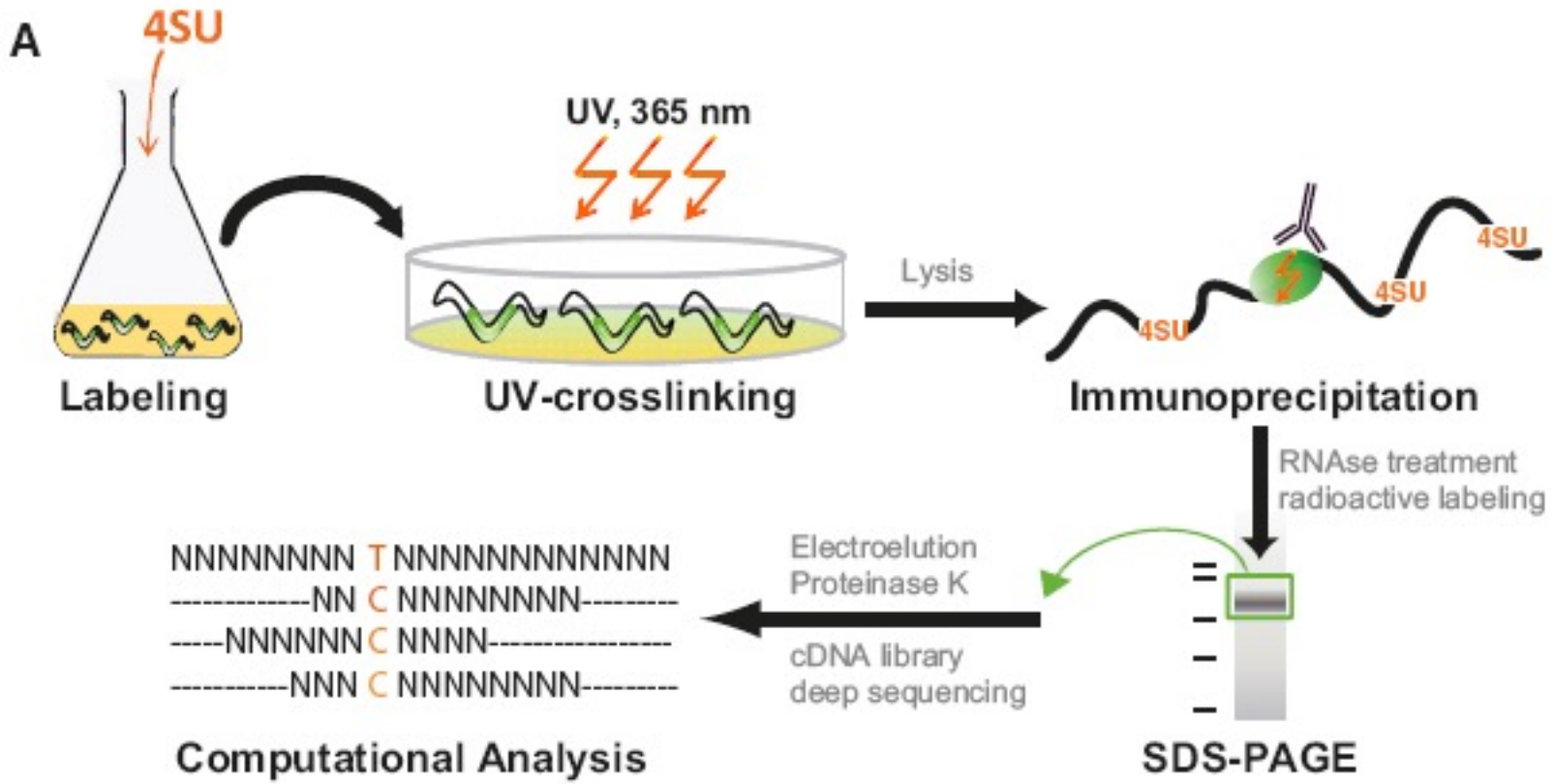


Creamer et al., PLOS Genet, 2011

Hafner et al., Cell, 2010

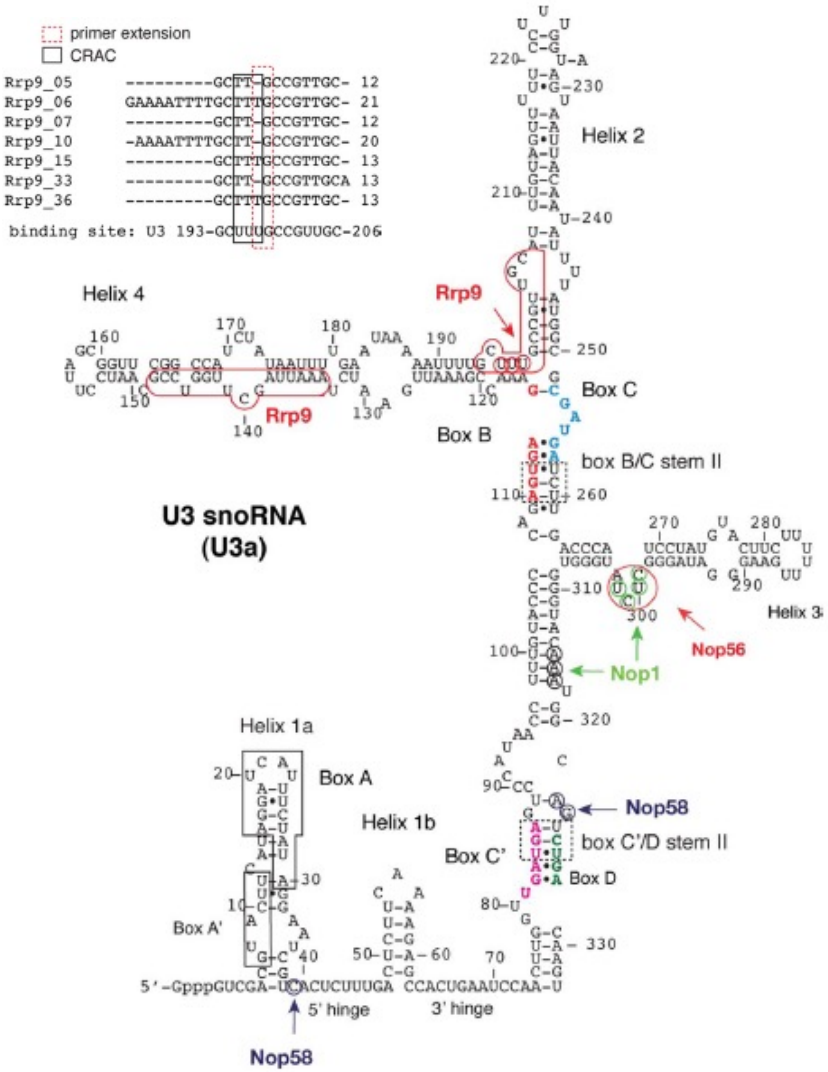
http://www.jove.com/index/details.stp?ID=2034

in vivo PAR-CLIP

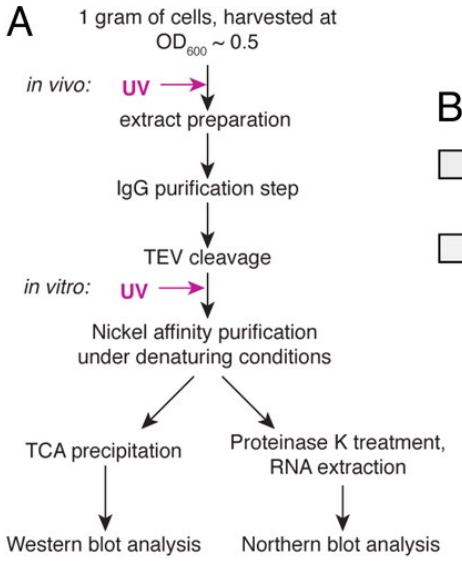


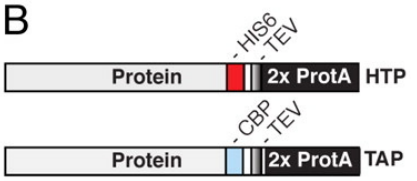
Jungkamp et al., Mol Cell, 2011

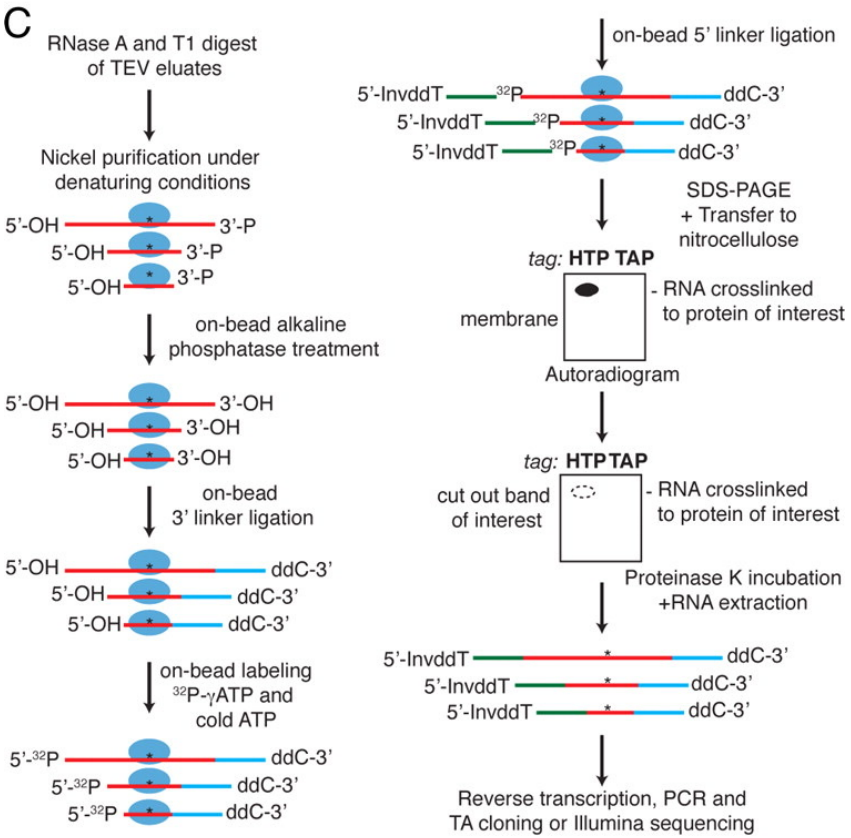
CRAC technique:
CRosslinking and Analysis of cDNA

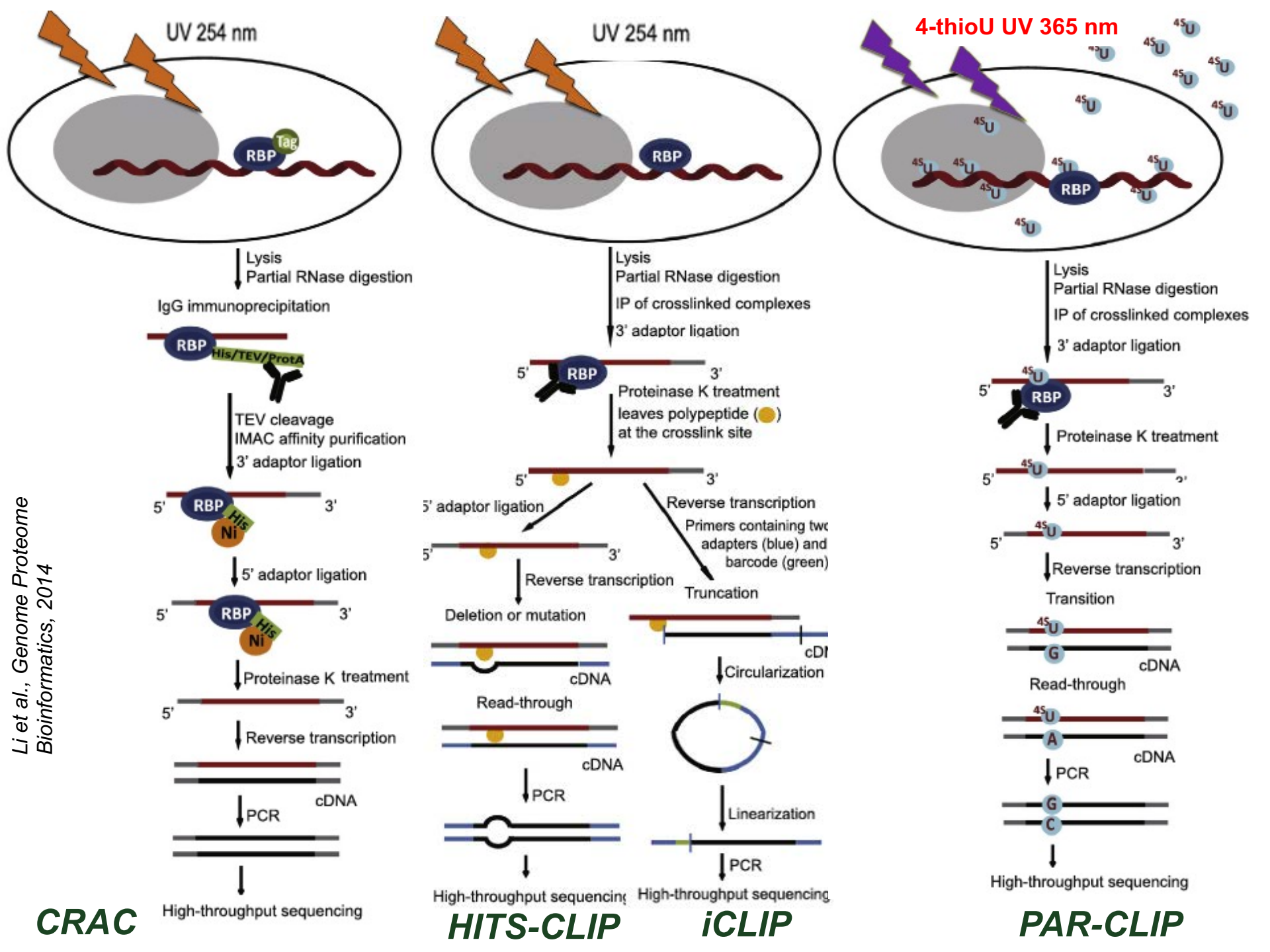


Granneman et al., PNAS, 2009



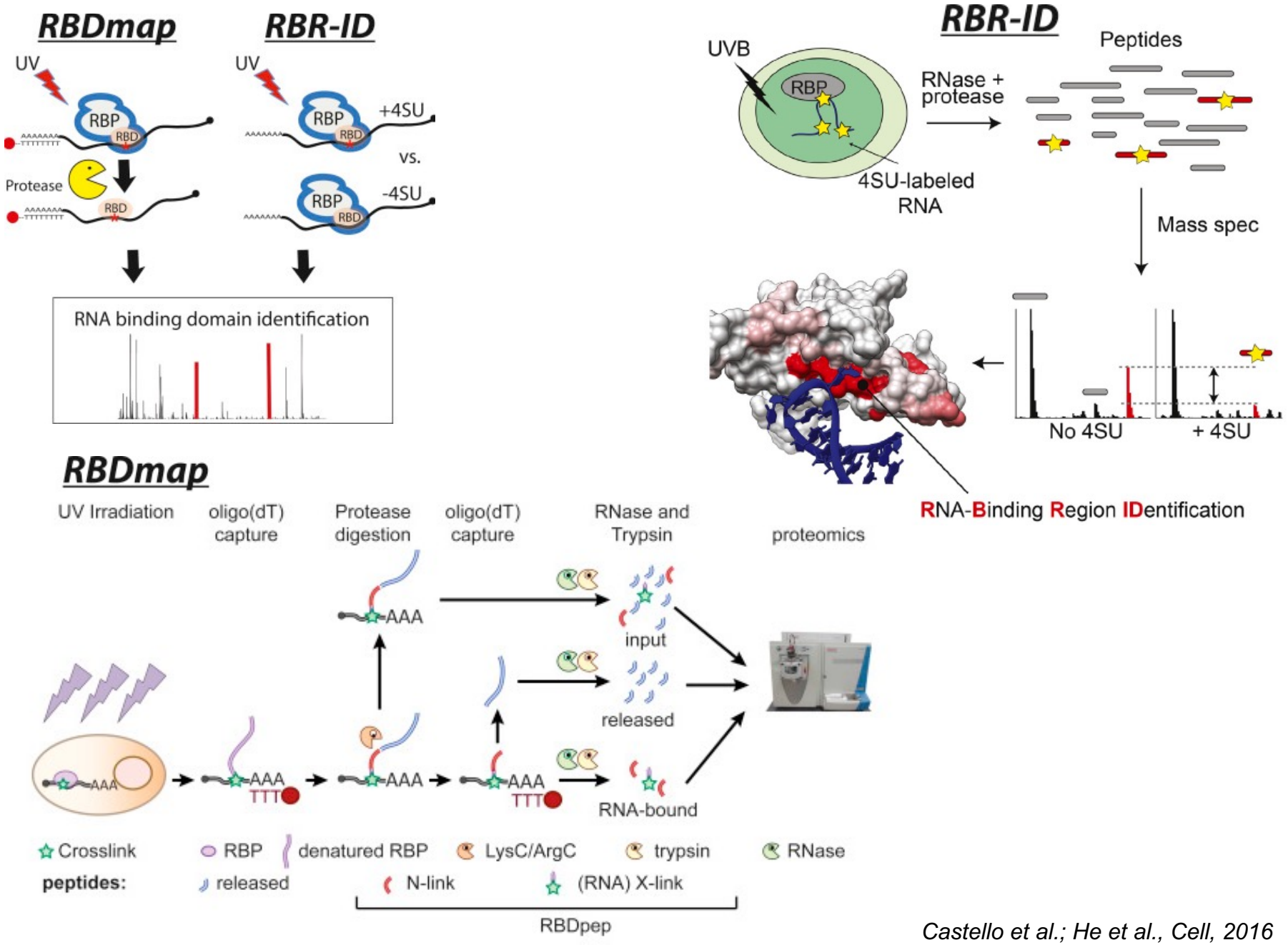






Li et al., Genome Proteome Bioinformatics, 2014

mRNA binding proteome (poly(A) BP)

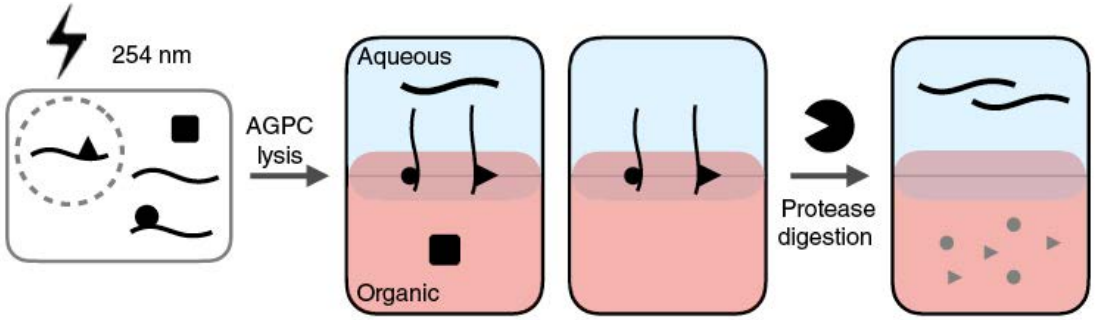


Castello et al.; He et al., Cell, 2016

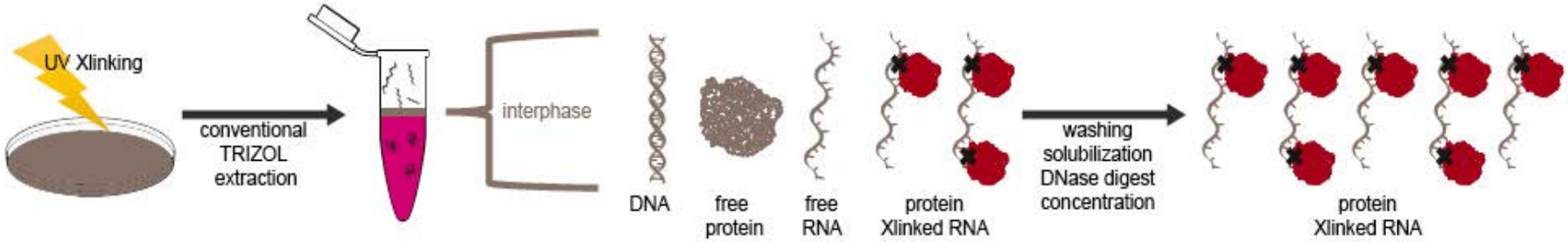
OOPS, XRNAX, TRAPP RNP interactome, RPBome

OOPS - orthogonal organic phase separation **XRNAX**
TRAPP/PAR-TRAPP - RNA-associated protein purification

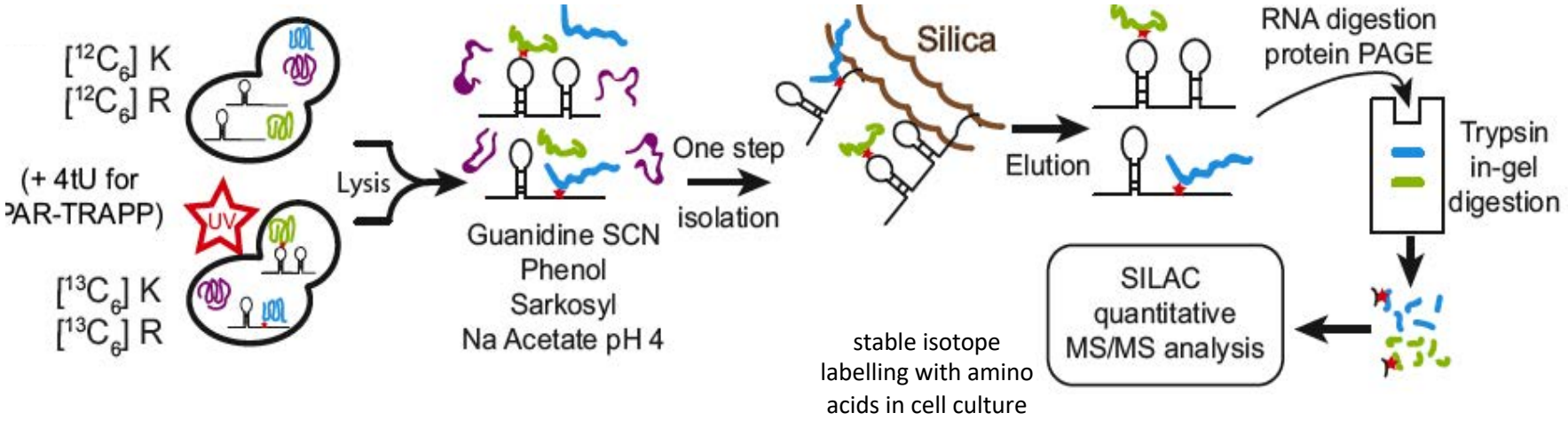
OOPS



XRNAX

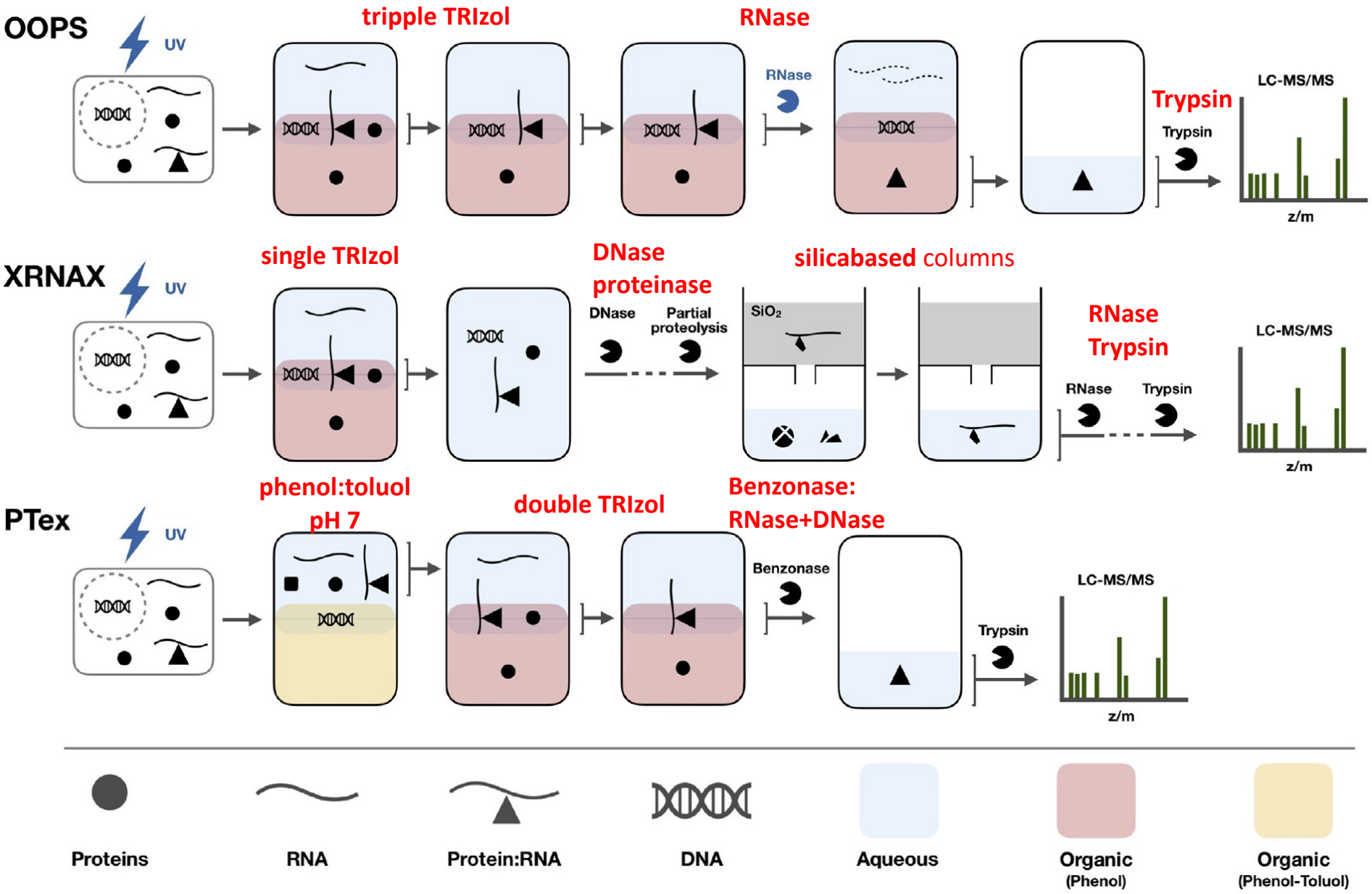


TRAPP/PAR-TRAPP



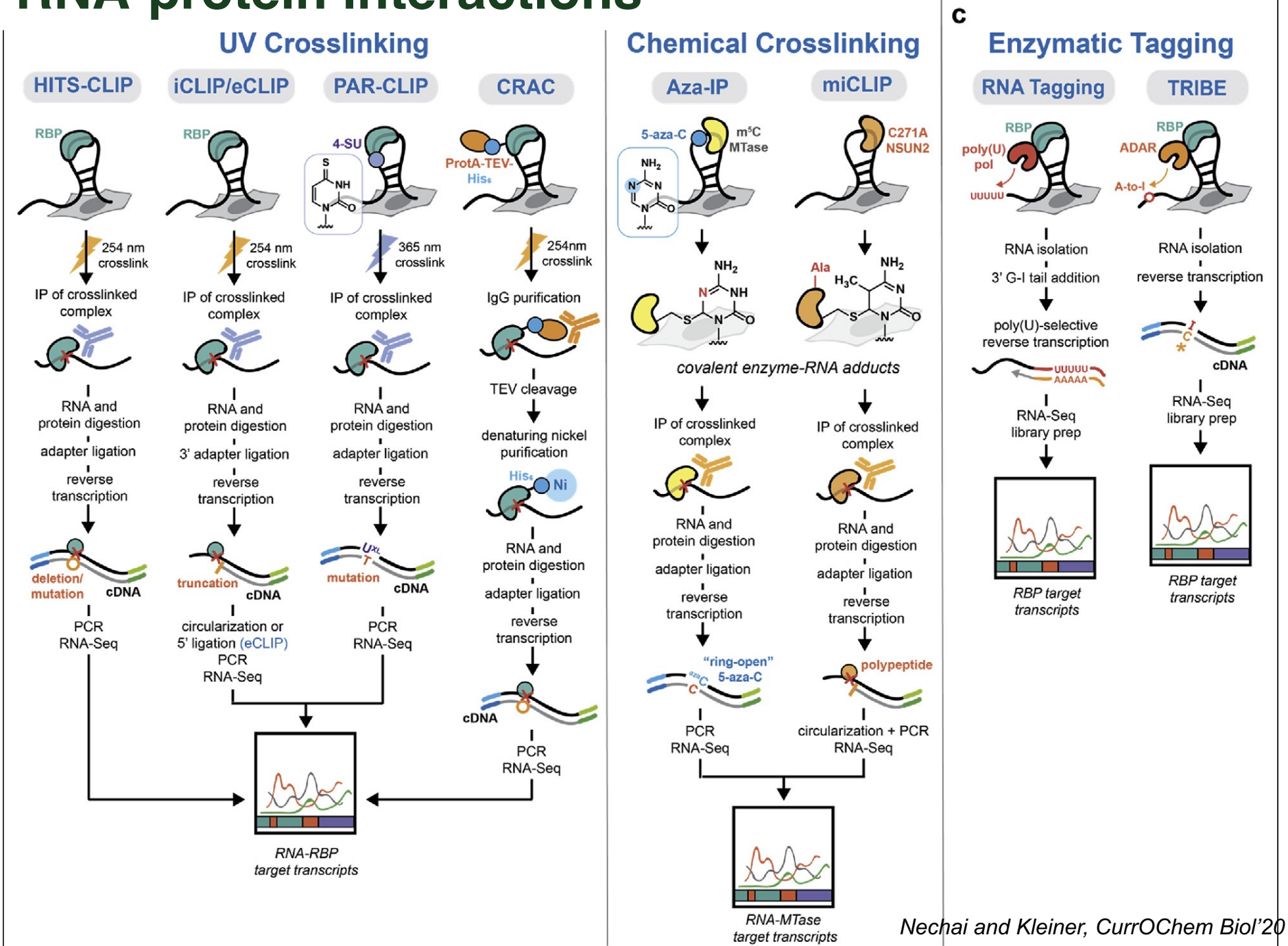
Queiroz et al, Nat Biotech, 2019; Shchepachev et al, Mol Sys Biol, 2019

OOPS, XRNAX, PTex – organic phase separation



Smith et al. Curr Op Chem Biol, 2020

RNA-protein interactions

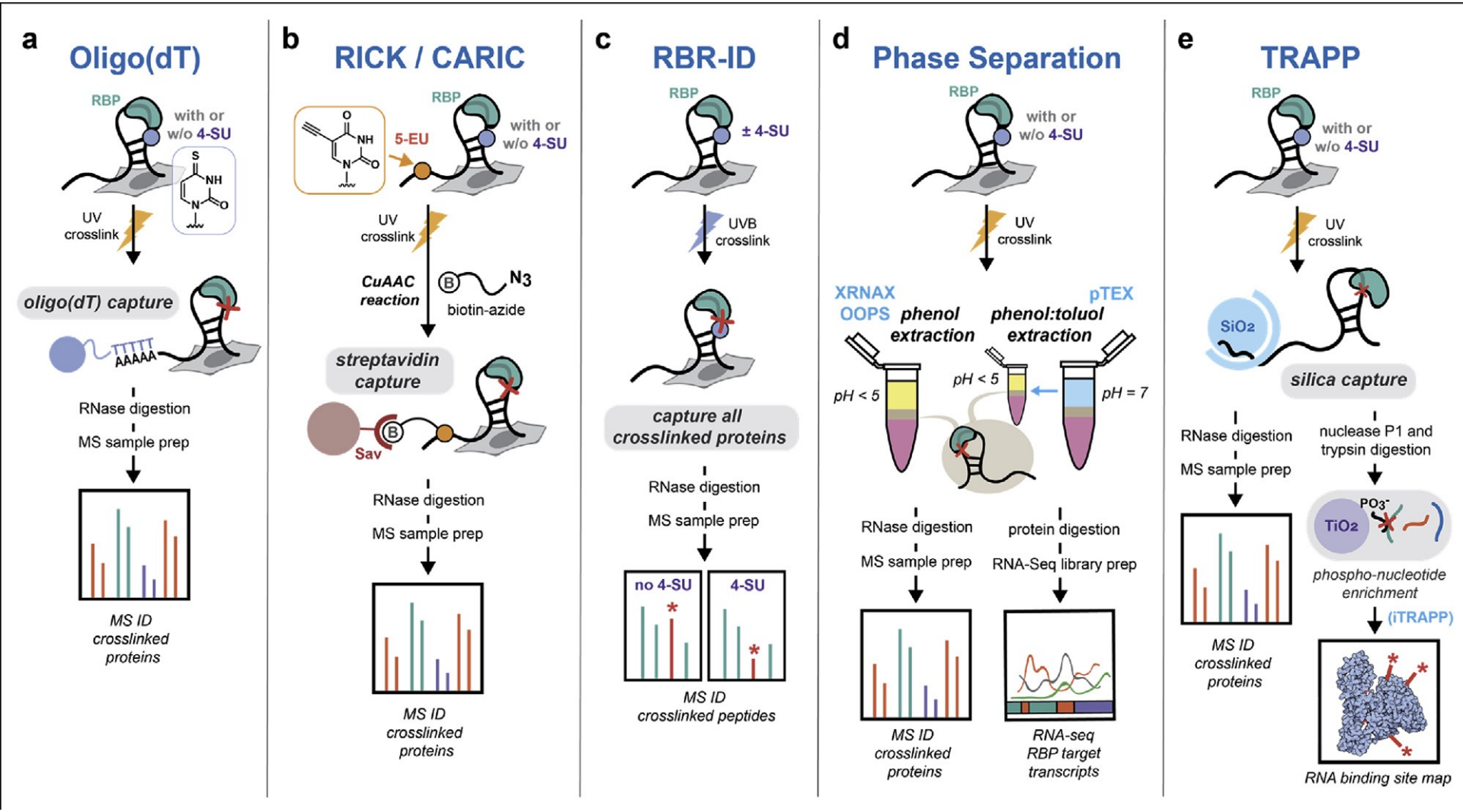


Nechai and Kleiner, CurrOChem Biol'20

RNA-protein interactions

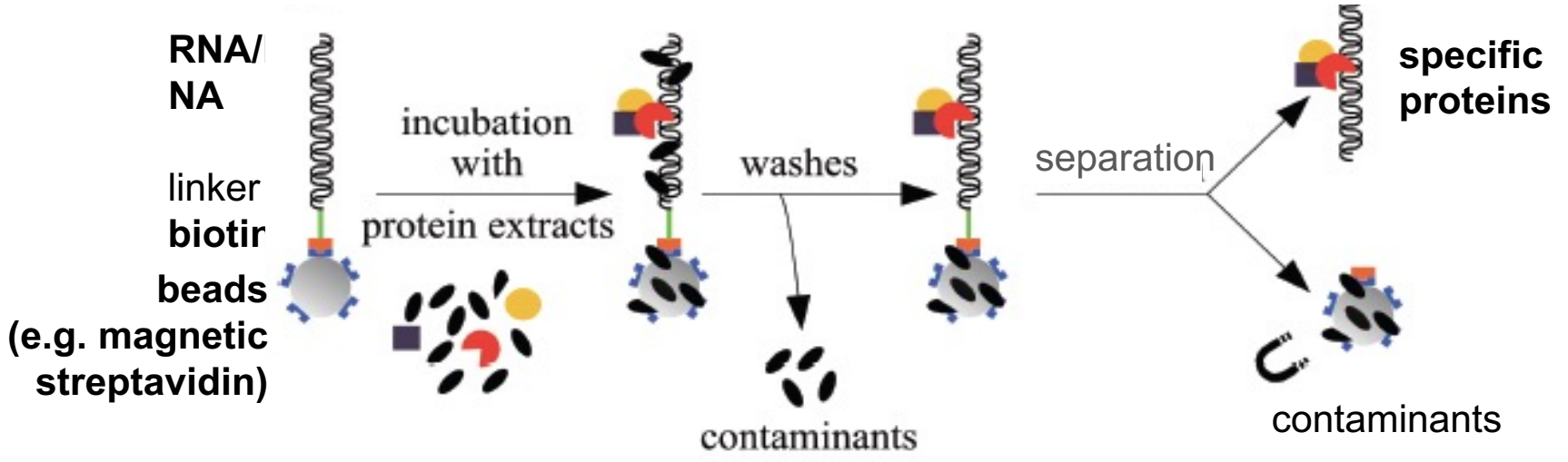
Nascent RNA can be labeled with 4-thioU (4-SU) or 6-SU 6-thioG

RICK/CARIC: with 5-ethynylU (5-EU), biotin is added to RNA by click chemistry for streptavidin capture

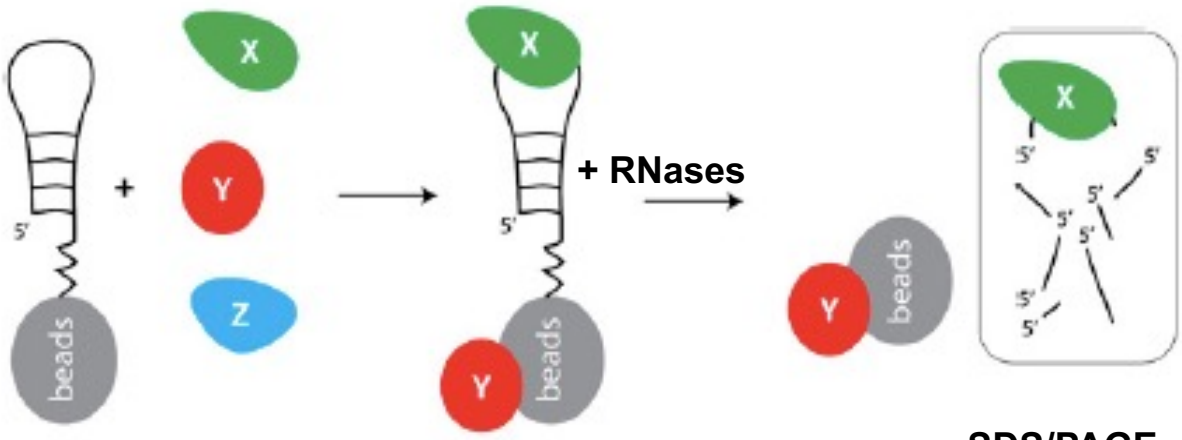


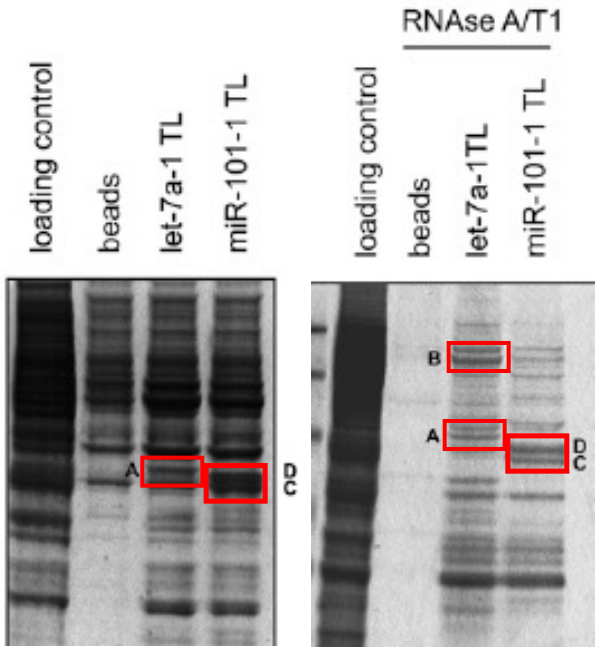
Nechai and Kleiner, CurrOChem Biol'20

RNA CHROMATOGRAPHY *in vitro*



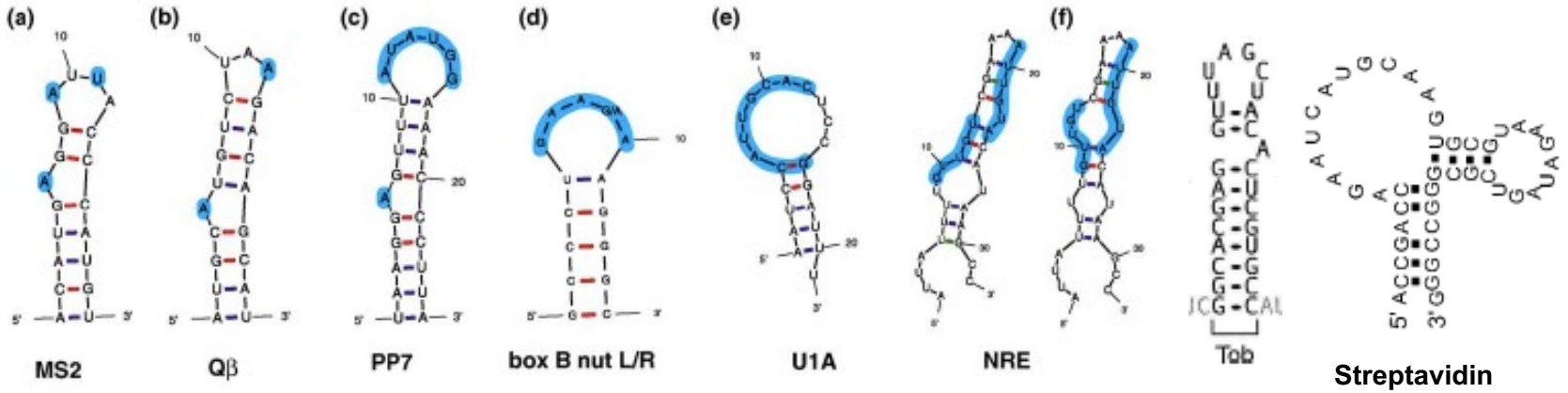
RNase-assisted RNA chromatography



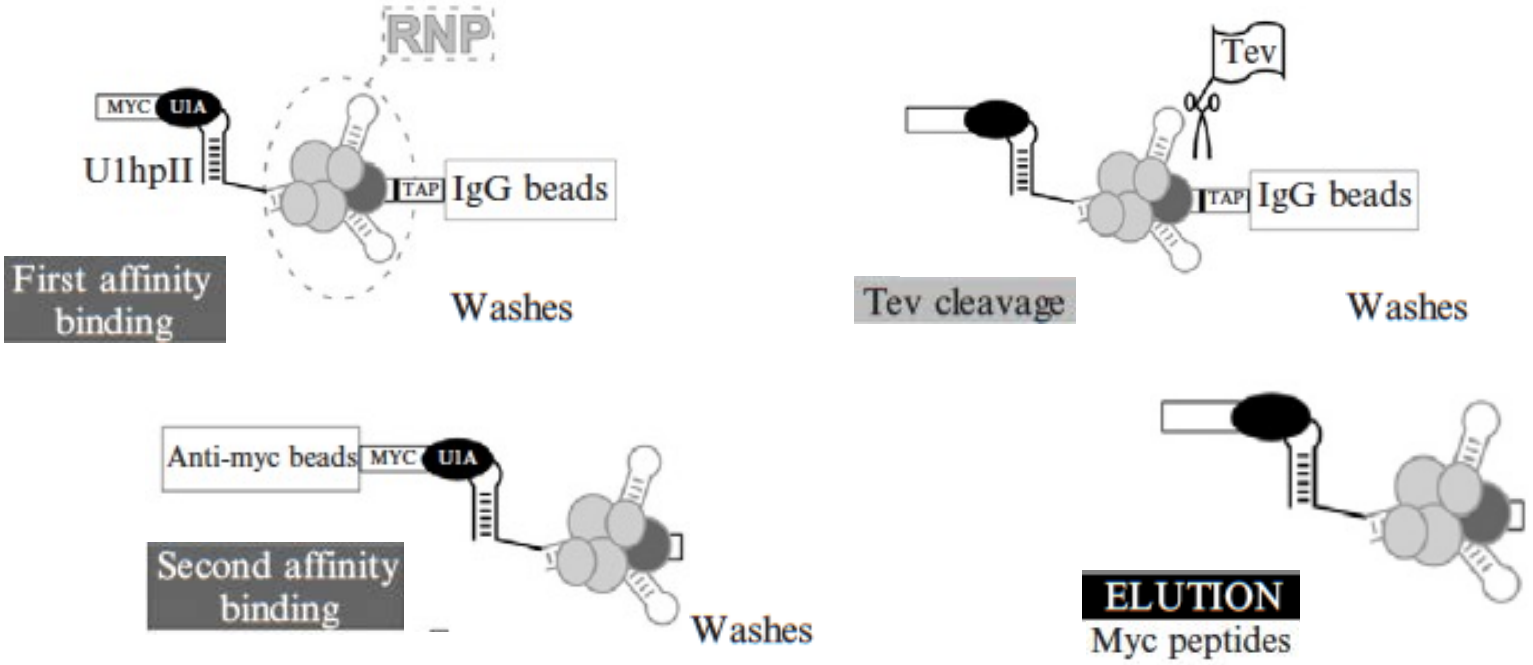


Hegarat et al., NAR, 2010; Michlewski and Caceres, RNA, 2010

RNA CHROMATOGRAPHY *in vivo*

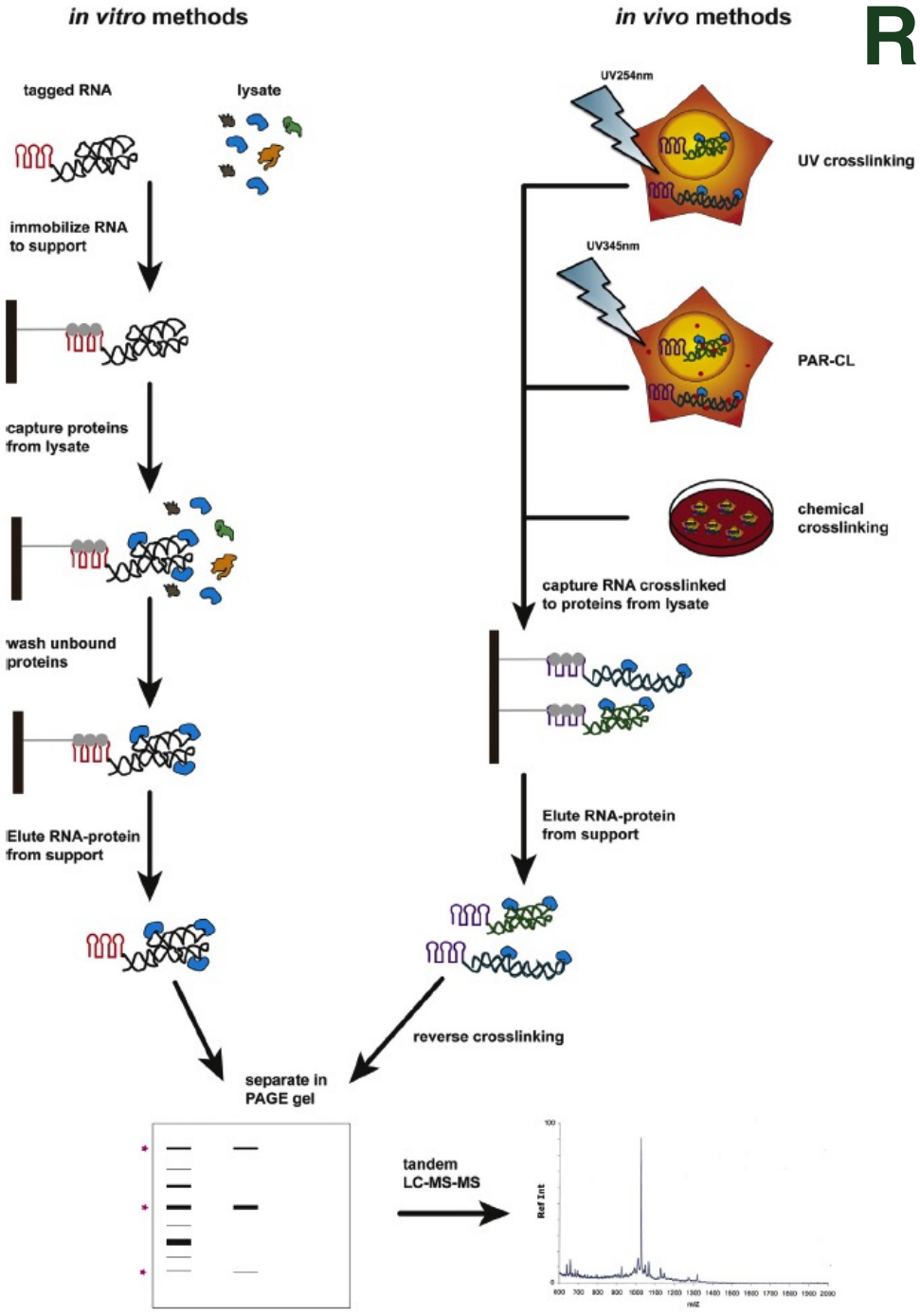


RNP purification from cells expressing RNA with affinity tags

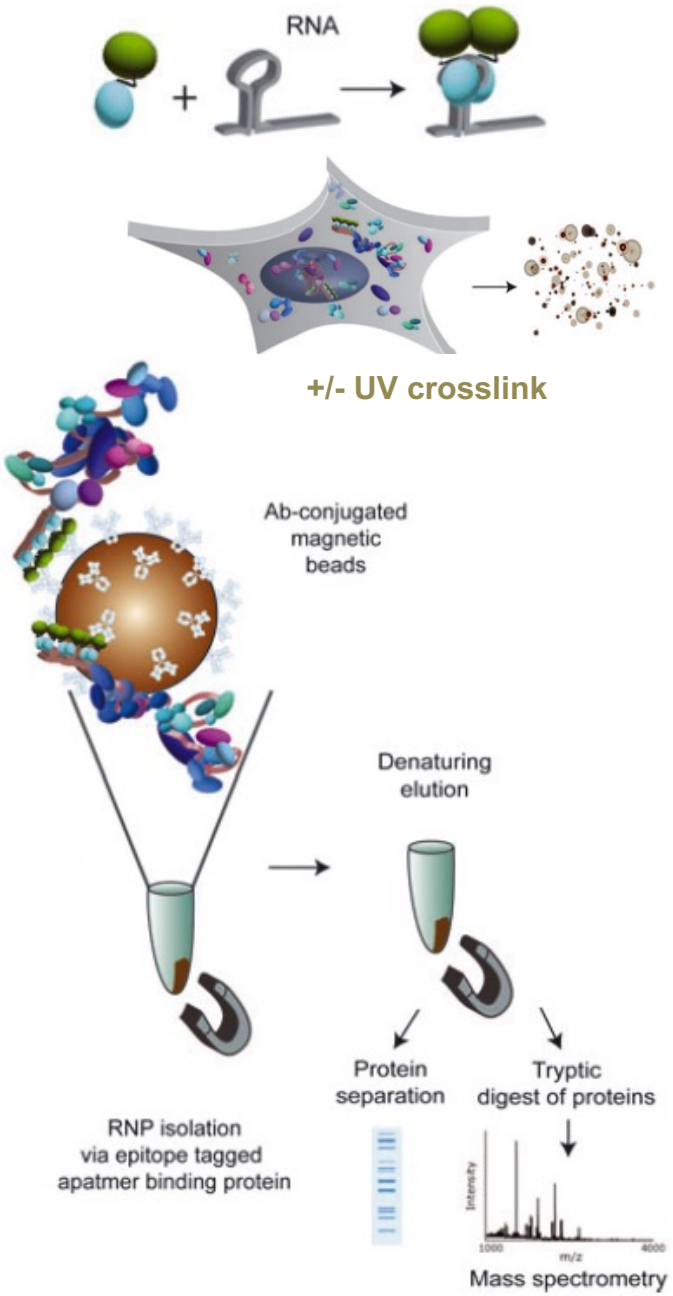


Higg and Collins, RNA, 2007; Srisawat and Engelke, Methods, 2002; Bachler et al., RNA, 1999; Weil et al., TiCB, 2010; Piekna-Przybylska et al., Meth Enzymol, 2007

RNA chromatography



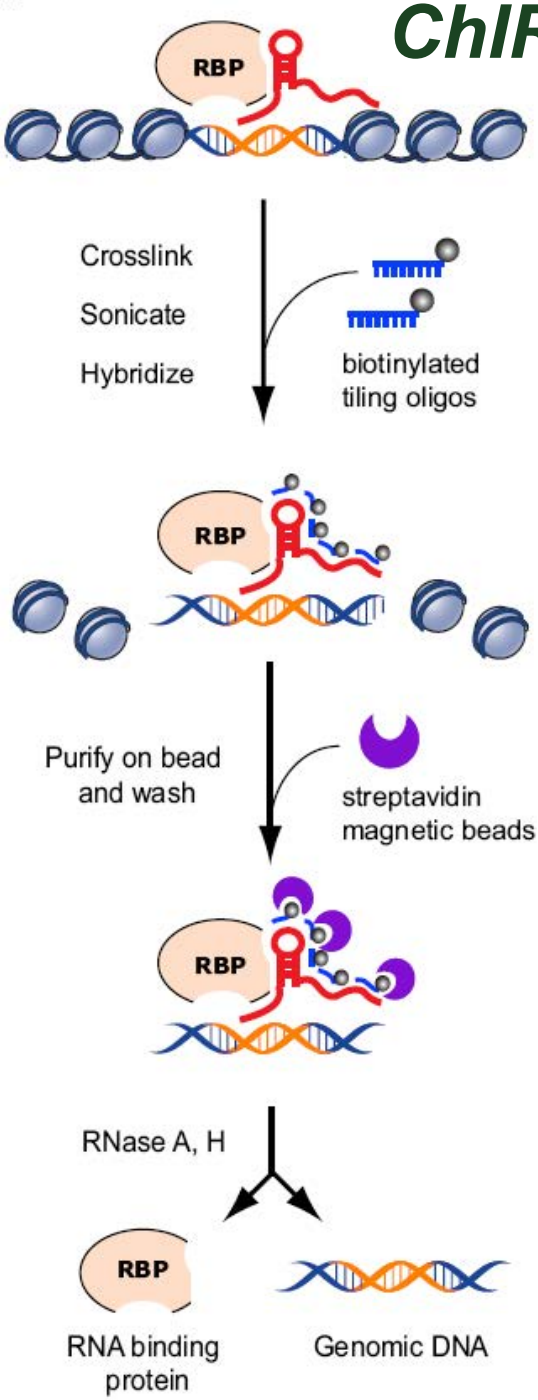
Faoro and Ataide, FEBS Lett, 2014



Oeffinger, Proteomics, 2012

Chromatin Isolation by RNA Purification

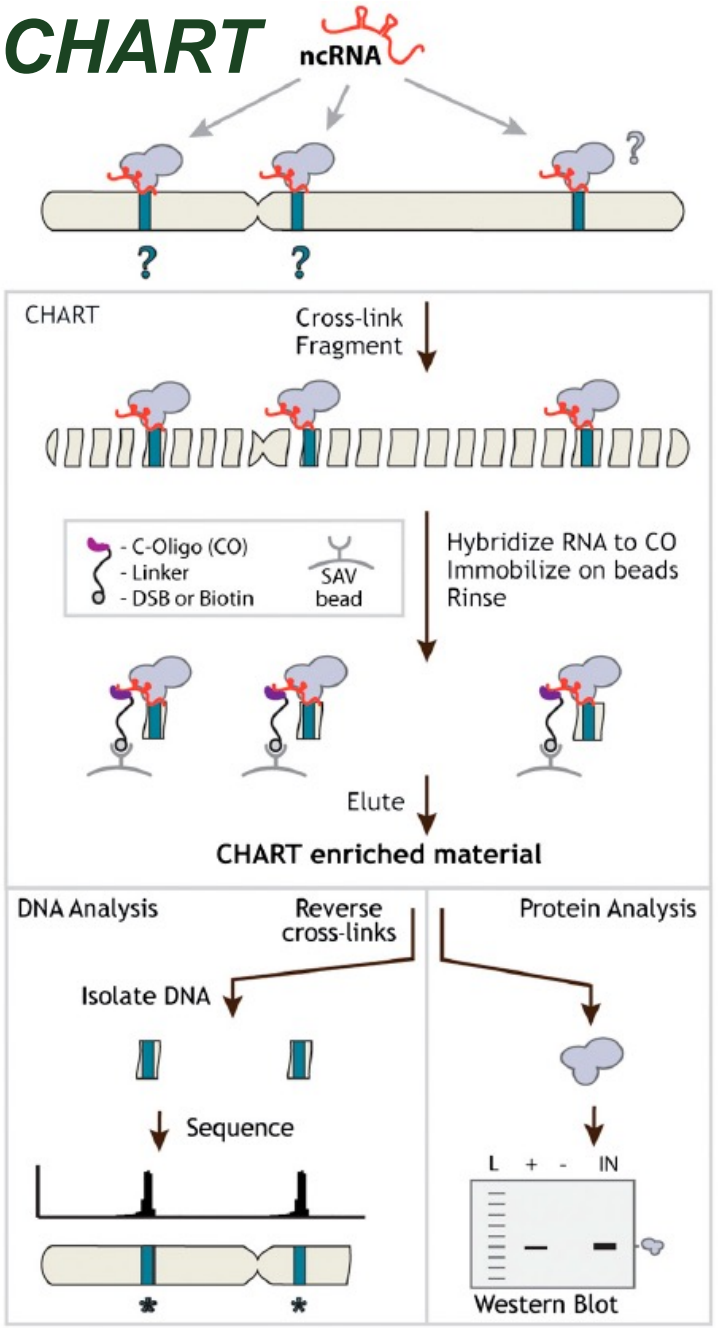
ChIRP



lncRNA
proteins
DNA

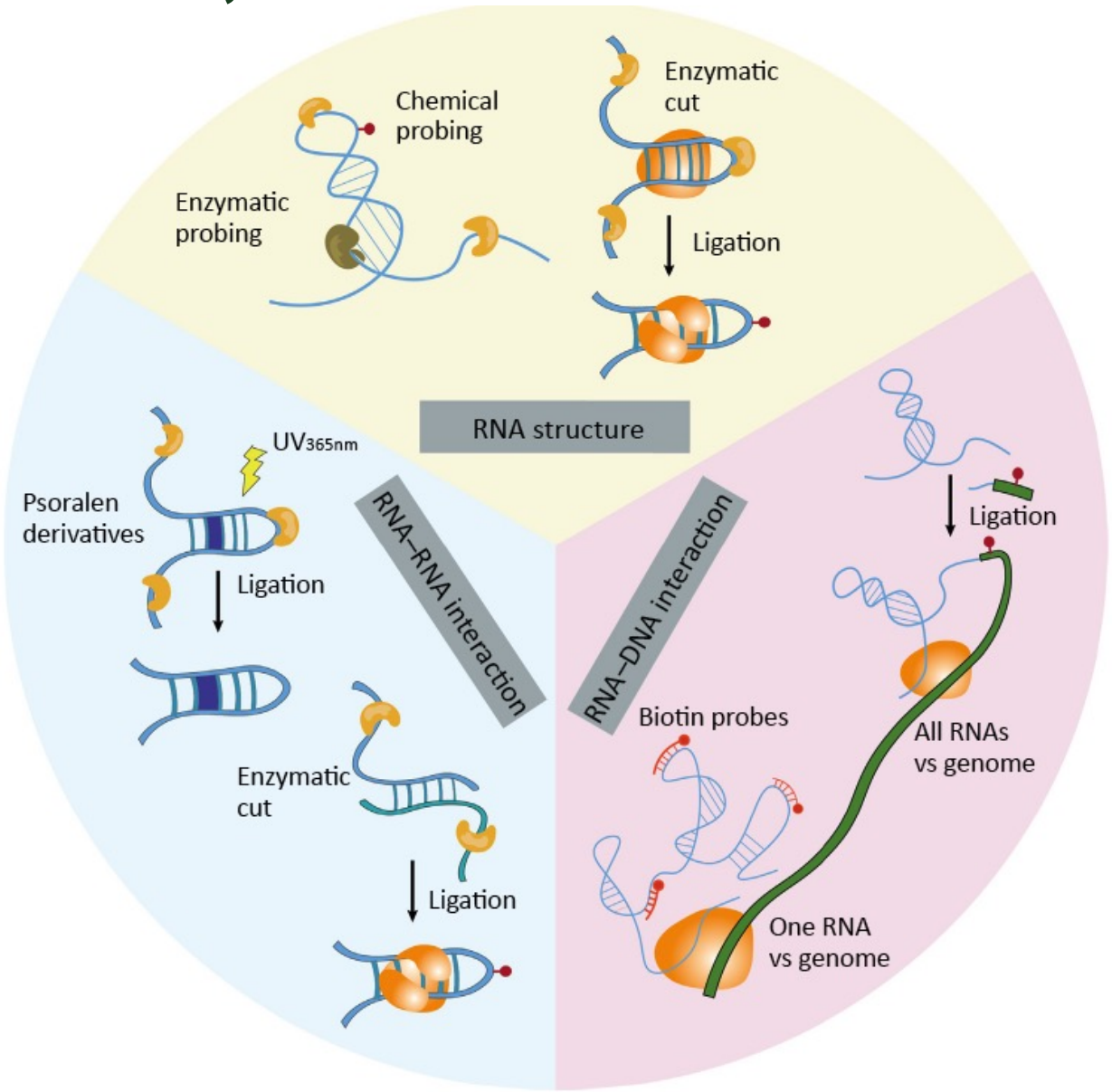
Capture Hybridization Analysis of RNA Targets

CHART



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

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

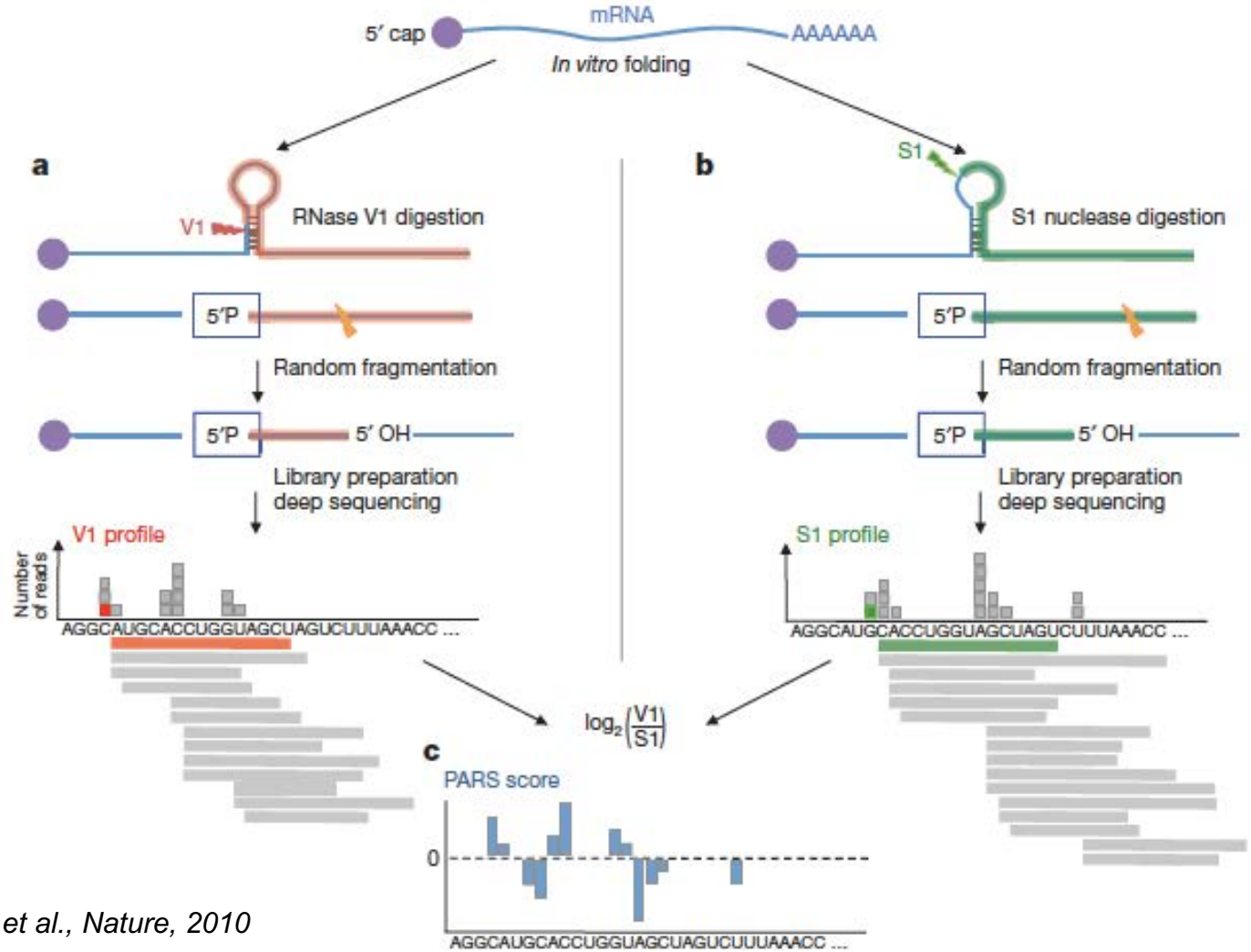


Nguyen et al, *TiG, 2018*

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

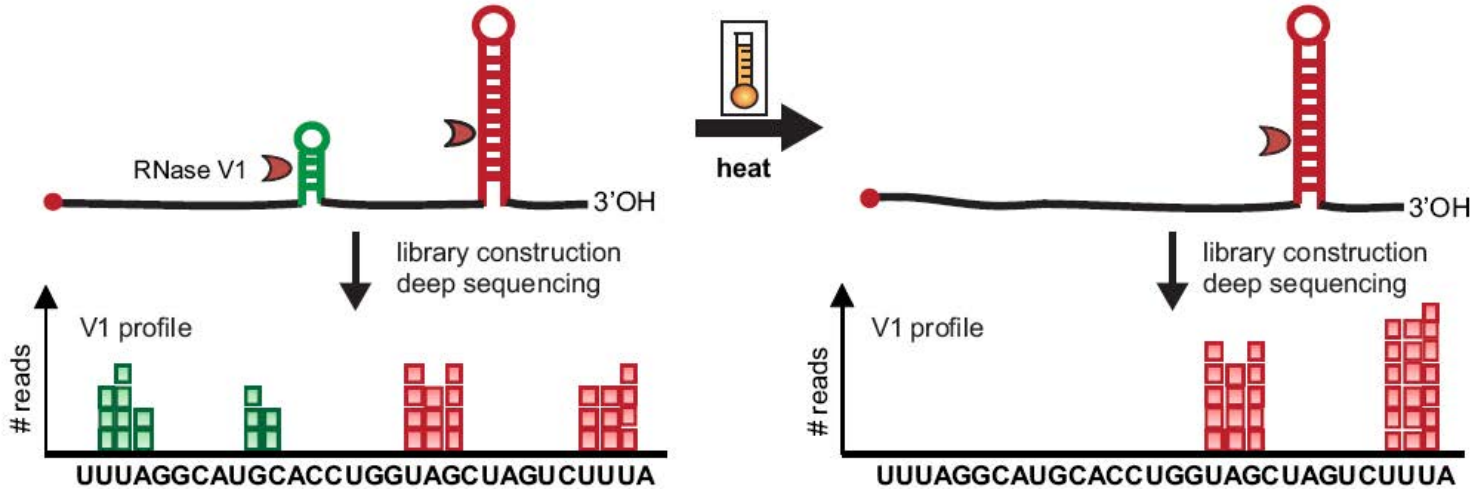
PARS: Parallel Analysis of RNA Structure

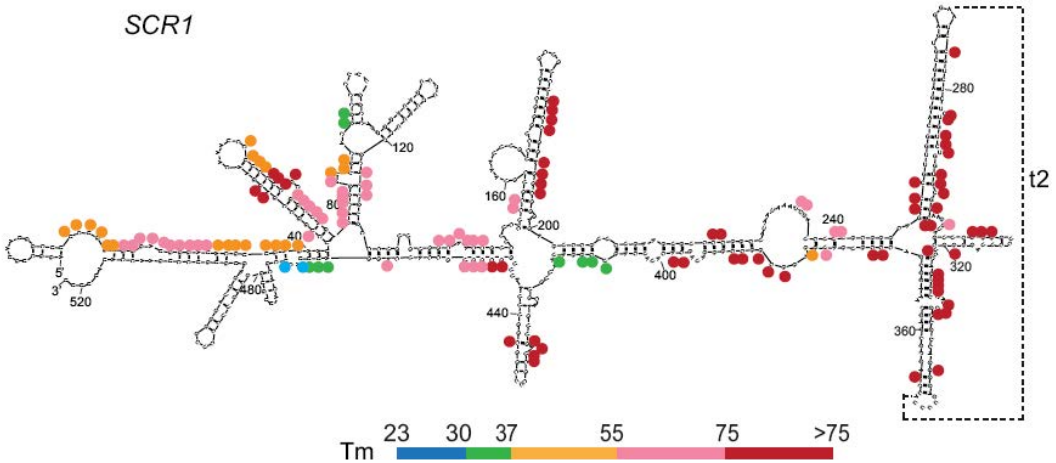
Molecular Cell
Article

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,*]





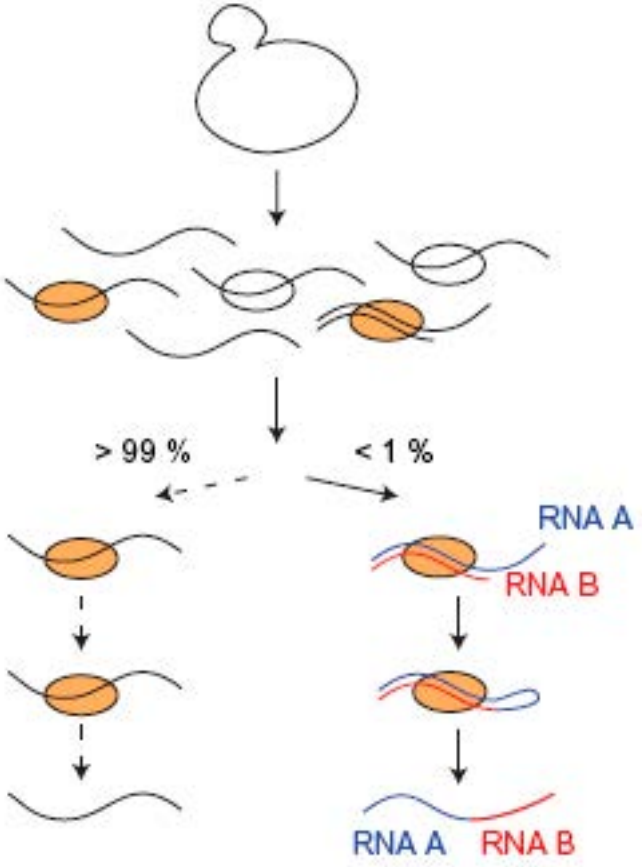
Regulatory Impact of RNA Secondary Structure across the *Arabidopsis* Transcriptome

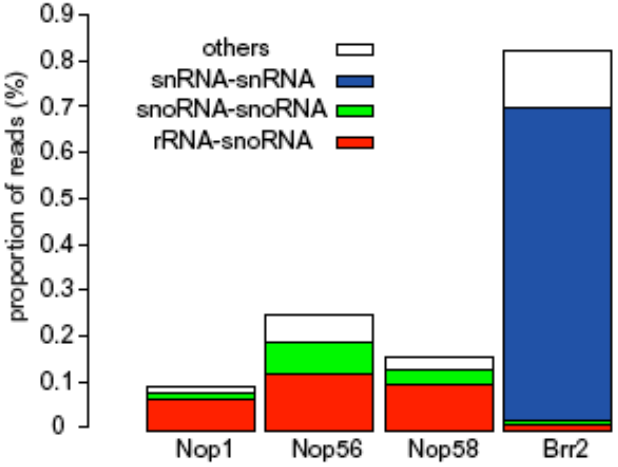
The Plant Cell 2012

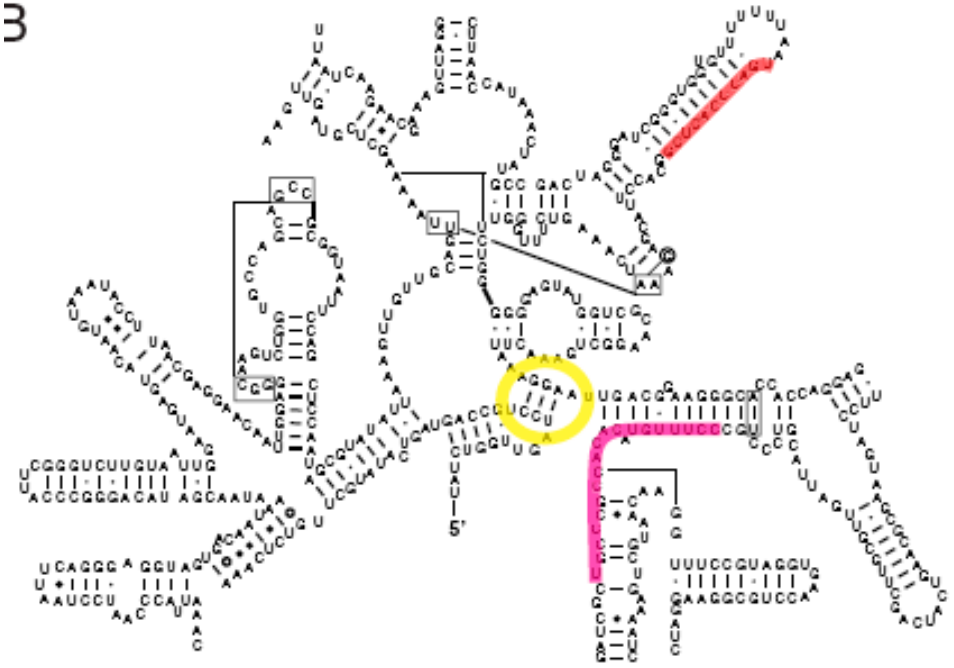
Fan Li,[a,b,c,1] Qi Zheng,[a,b,1] Lee E. Vandivier,[a,b,d] Matthew R. Willmann,[a,b] Ying Chen,[a,b,c] and Brian D. Gregory[a,b,c,d,2]

CLASH (intra- and intermolecular RNA-RNA interactions)

Crosslinking Ligation and Sequencing of Hybrids





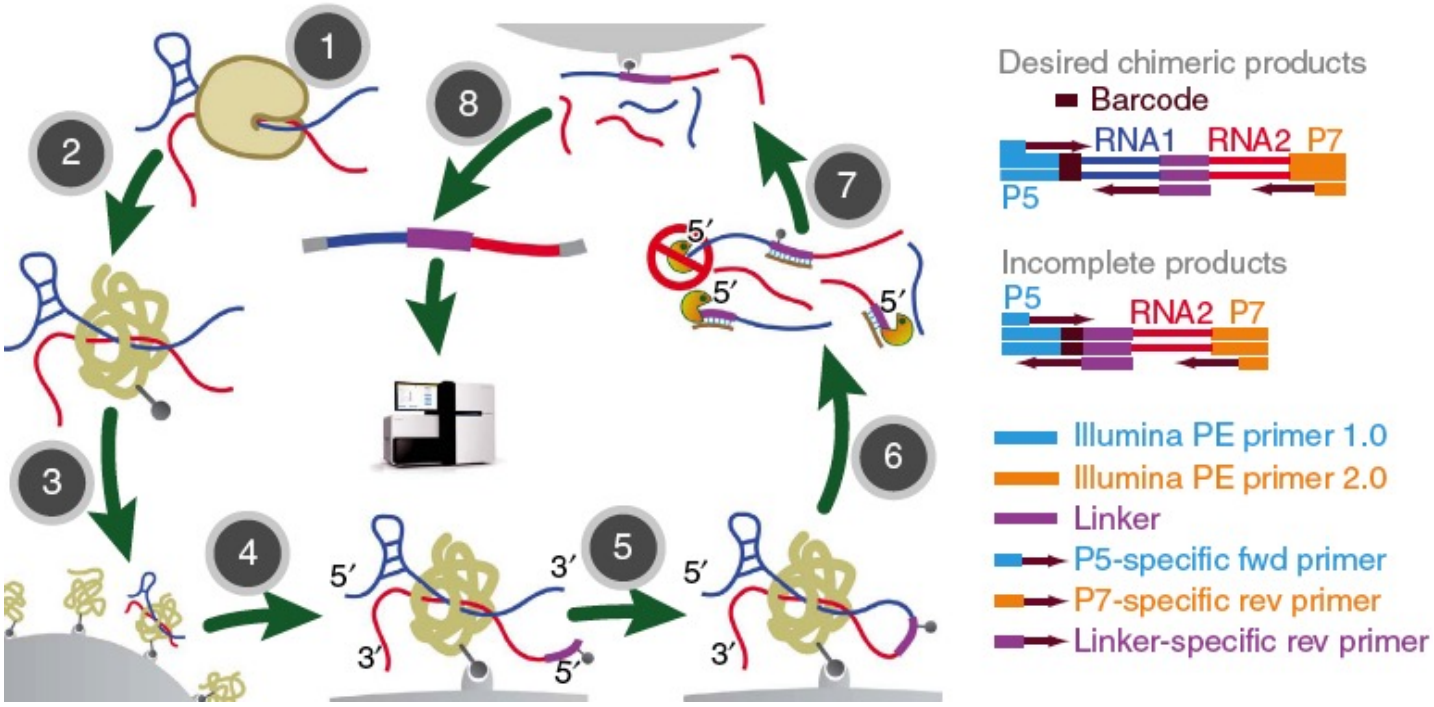


U3- 18S rRNA interactions

Kudla et al., PNAS, 2011

MARIO (intra- and intermolecular RNA-RNA interactions)

Mapping RNA interactome in vivo



Nguyen et al., NatComm, 2016

(1) cross-linking RNAs to proteins
(2) RNA fragmentation, protein denaturing and biotinylation
(3) immobilization of RNA-binding proteins via biotin at low density
(4) ligation of a biotinylated RNA linker to RNA 5' end
(5) proximity ligation under dilute conditions
(6) removal of unligated RNA by RNase H activity of T7 exonuclease
(7) reversal of crosslink, protein removal, RNA purification
(8) biotin pull-down of chimeric RNA with biotinylated linker, (9) library construction

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

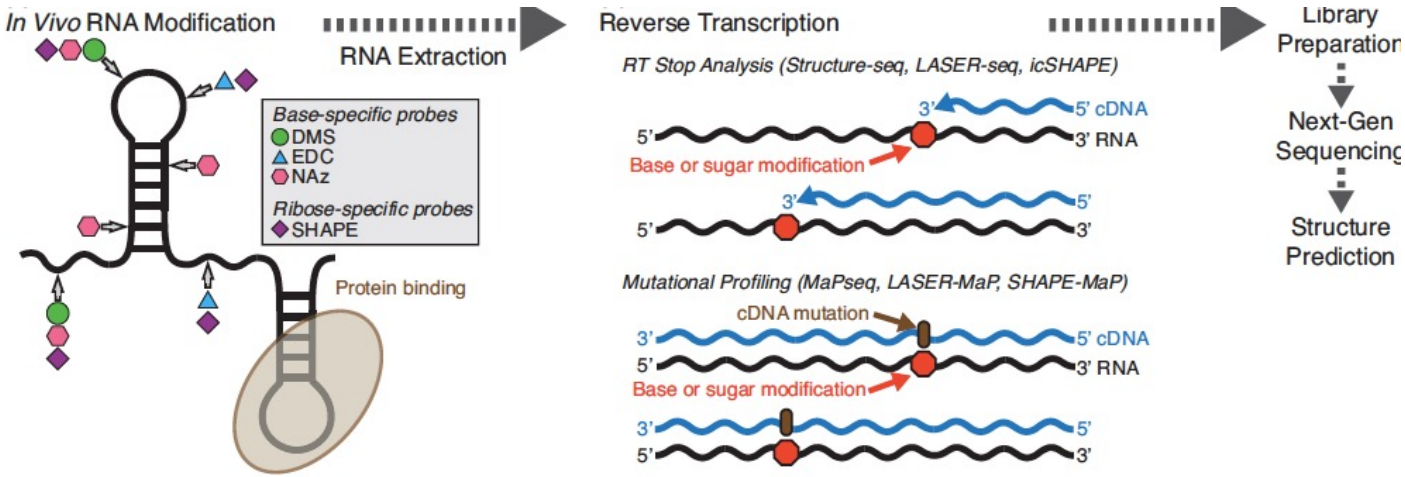
SHAPE chemicals: DMS, dimethyl sulfate; 1M7, 1-methyl-7-nitroisatoic anhydride
SHAPE enzymes: P1 nuclease, RNases V1 and S1
PARIS/SPLASH chemicals: psoralen; AMT, 4'-aminomethyltrioxsalen

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

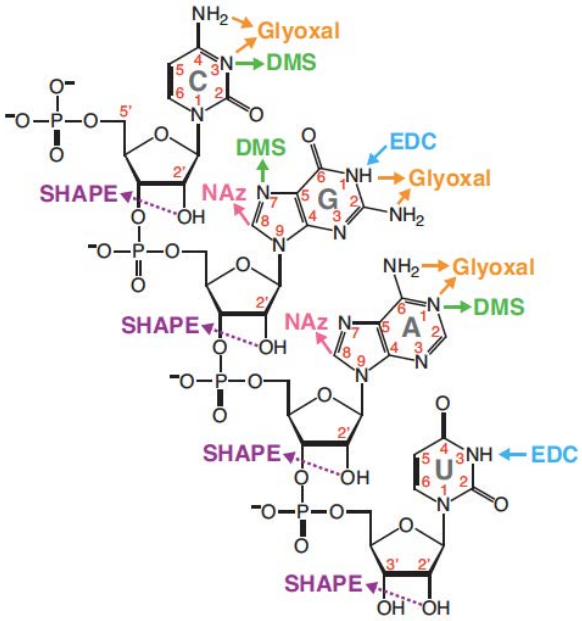
Graveley, Mol Cell, 2016

MaP, SHAPE, SHAPE-MaP, RING-MaP, Mod, CRIS etc...



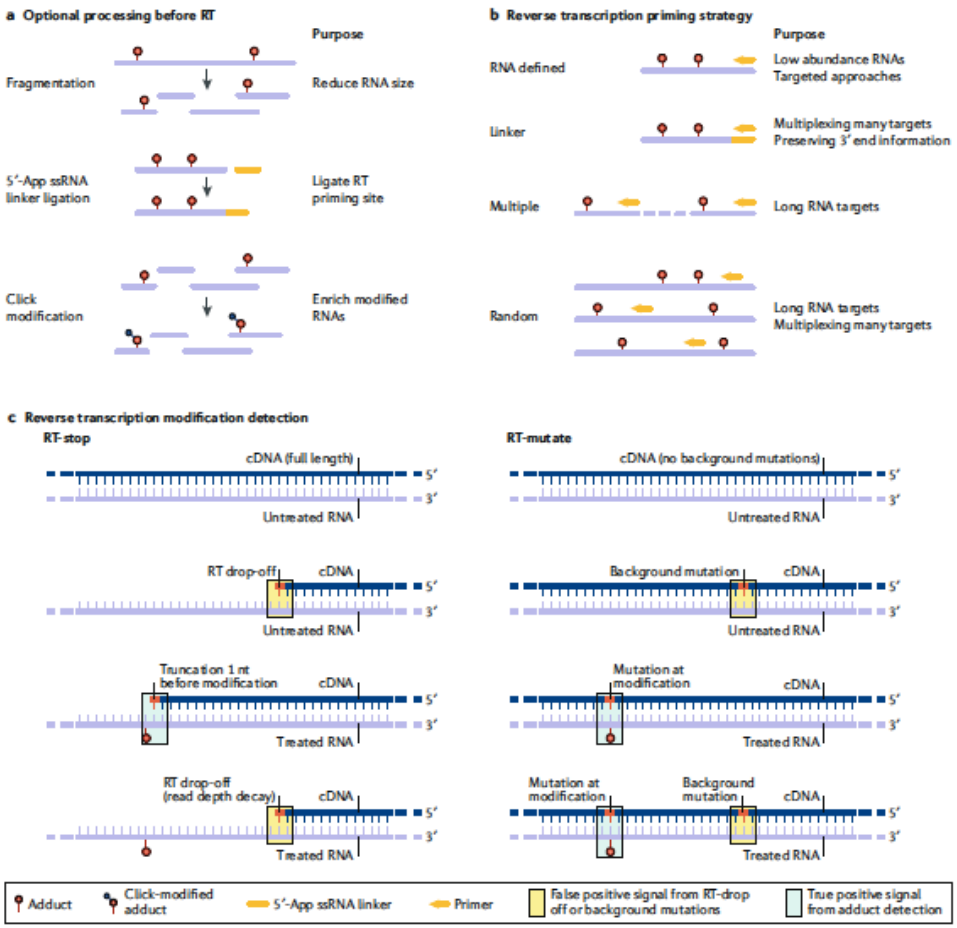
RNA structure

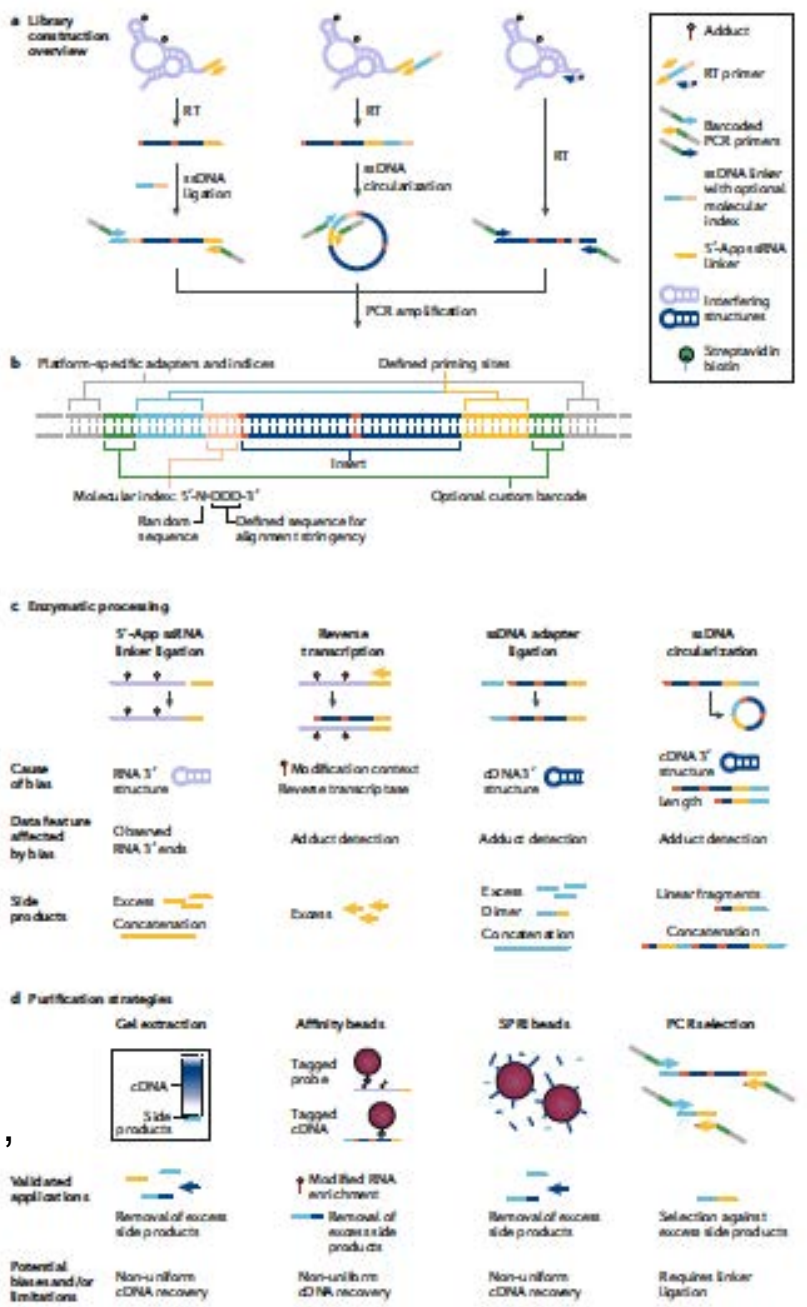
	Probe	Primary modification sites
SHAPE	*N*-methylisatoic anhydride (NMIA)	2′ OH of all nts
	1-methyl-7-nitroisatoic anhydride (1M7)	2′ OH of all nts
	1-methyl-6-nitroisatoic anhydride (1M6)	2′ OH of all nts
	Benzoyl cyanide (BzCN)	2′ OH of all nts
	2-methylnicotinic acid imidazolide (NAI)	2′ OH of all nts
	2-methyl-3-furoic acid imidazolide (FAI)	2′ OH of all nts
	2-(azidomethyl)nicotinic acid imidazolide (NAI-N_3)	2′ OH of all nts
Base pairing	Dimethyl sulfate (DMS)	G N7, A N1 and C N3
	N-cyclohexyl-*N*′-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT)	G N1 and U N3
	Kethoxal and other 1,2-dicarbonyl compounds	G N1 and C2-amine
Solvent accessibility	Hydroxyl radical (•OH)	Backbone
	Nicotinoyl Azide (NAz)	G C8 and A C8



Mitchel III et al, CurrOpStructBiol, 2019
Strobel et al, NatRevGenet, 2018

MaP, SHAPE, SHAPE-MaP, RING-MaP, Mod, CRIS etc...

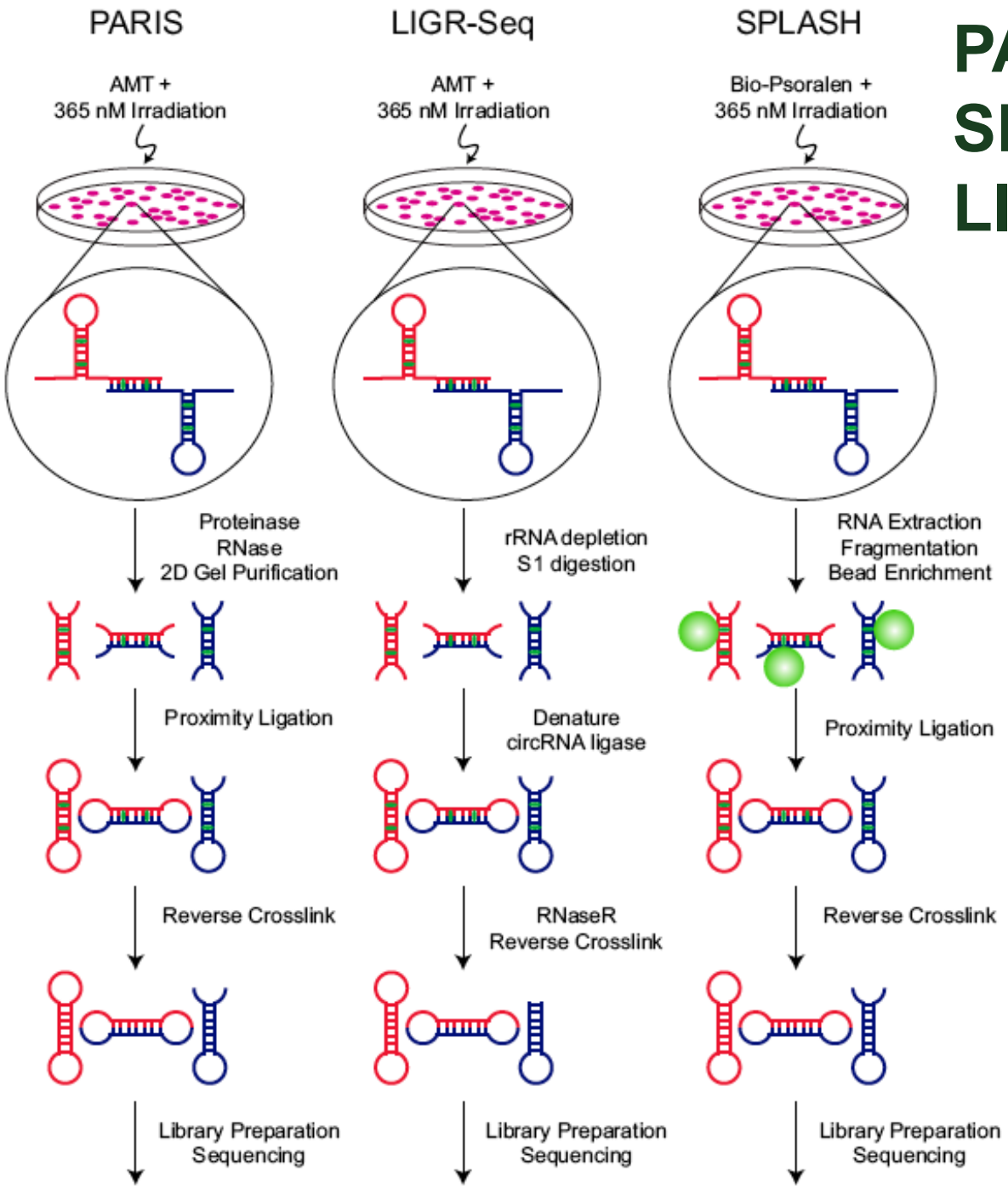




Strobel et al, NatRevGenet, 2018

- Additional modifications
- Reverse Transcription
- Detection of modifications
- Library construction: amplification, adapter ligation, circularization etc etc
- Purification
- Structure calculation

RNA structure



PARIS
SPLASH
LIGR

RNA structure

- 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

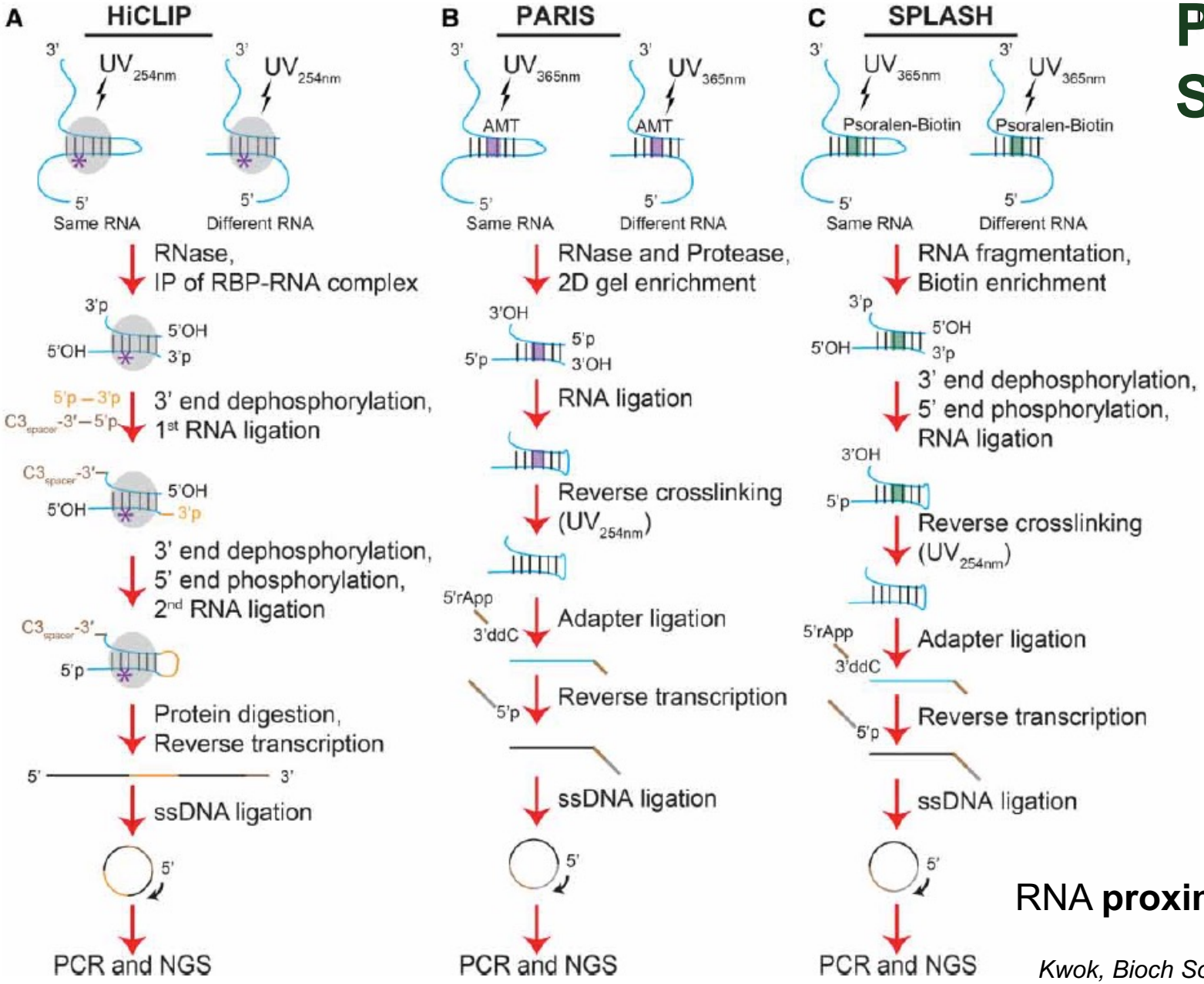
[AMT = psoralen derivative 4'-aminomethyltrioxalen] and

Graveley, Mol Cell, 2016

RNA structure

RNA-protein interactions

HiCLIP
PARIS
SPLASH



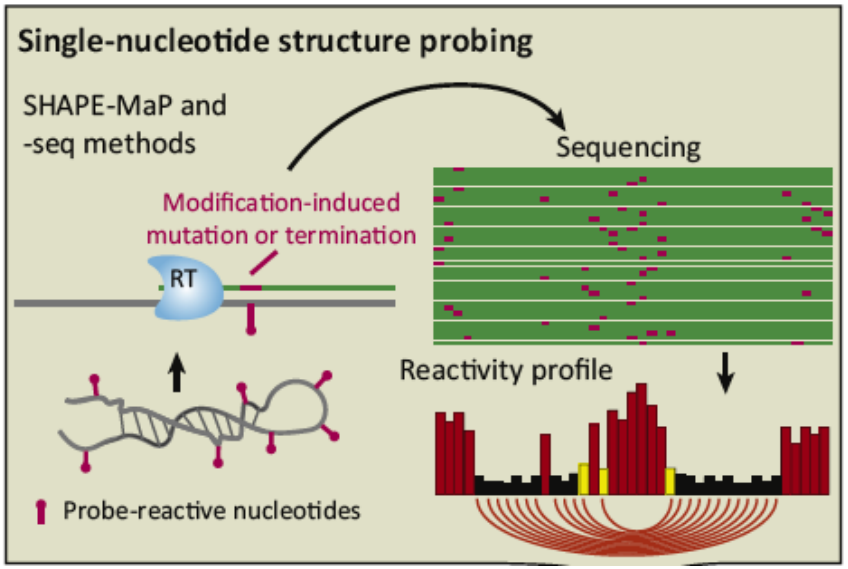
RNA **proximity ligation**

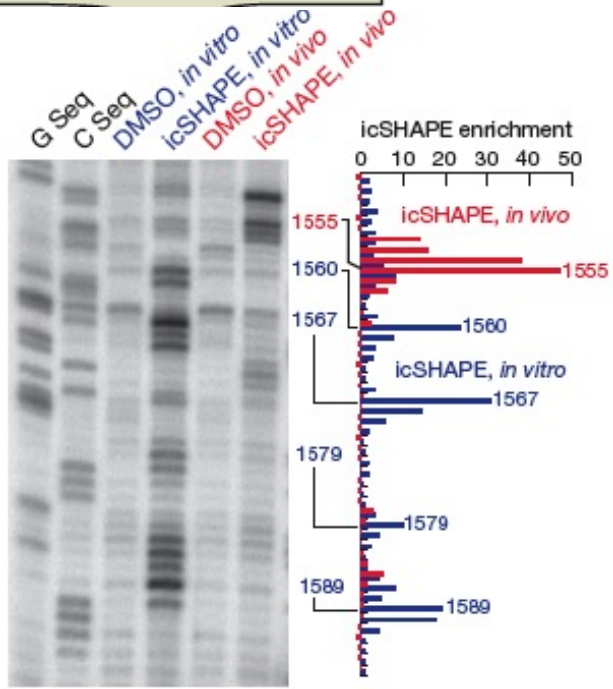
Kwok, Bioch Society Trans, 2015

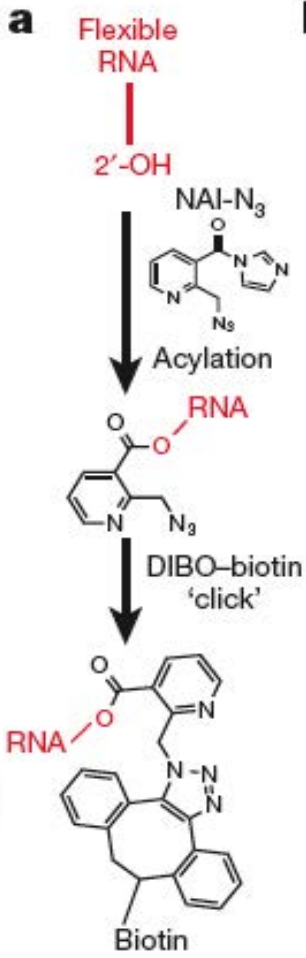
RNA structure *in vivo*: icSHAPE

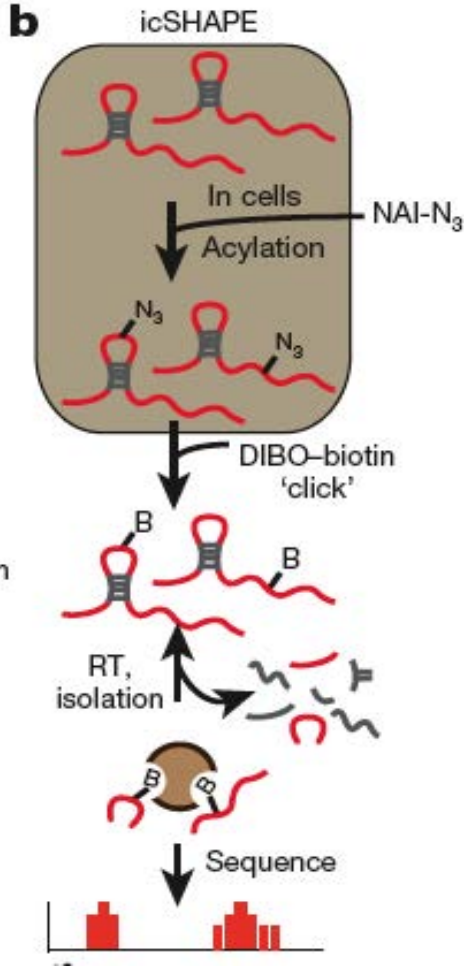
RNA structure

icSHAPE: click selective 2'-hydroxyl acylation and profiling



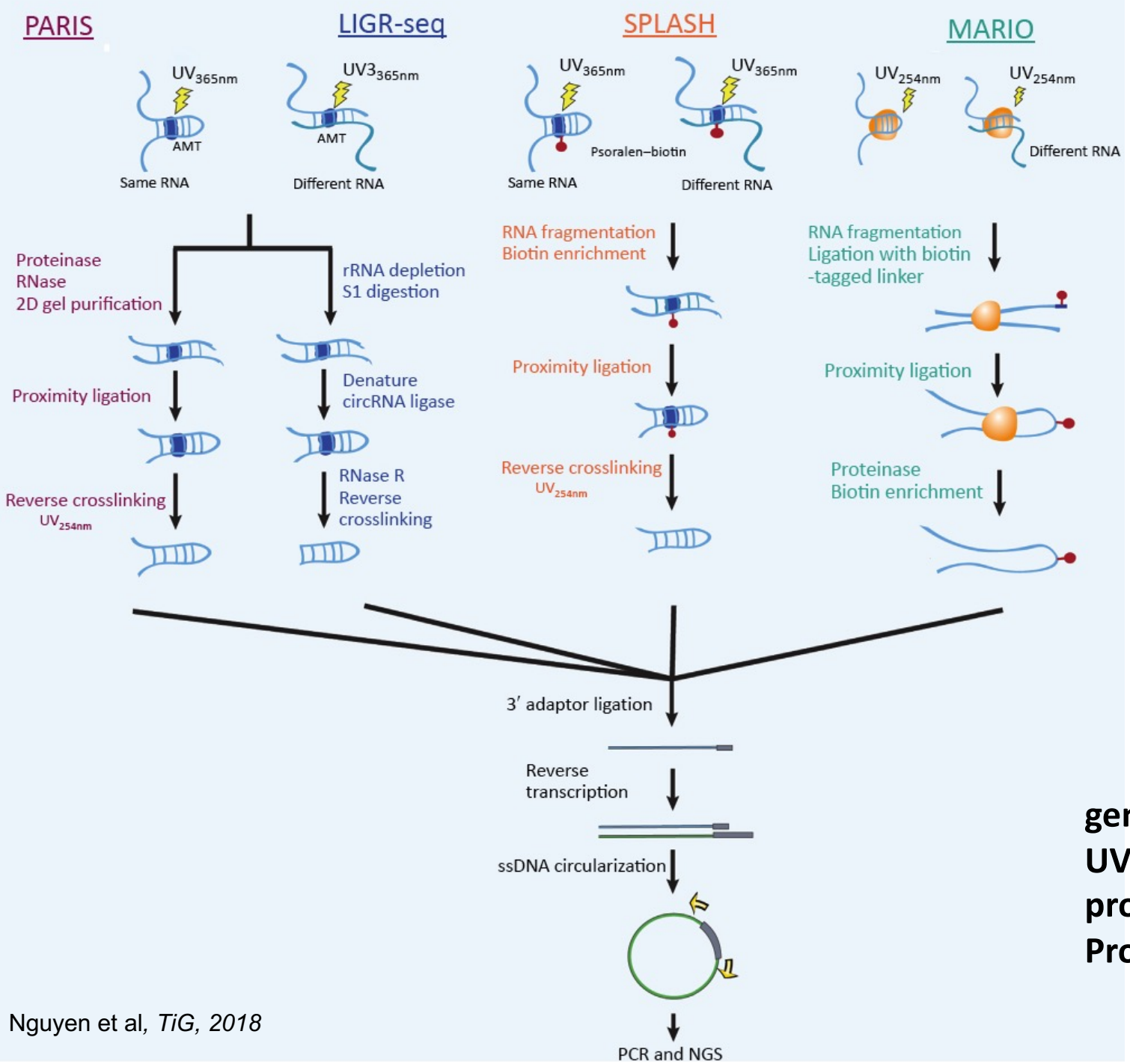






Low and Weeks, Methods 2010
Weidman et al, TiBS, 2016

RNA structure



genomewide
UV crosslink
protein-mediated
Proximity ligation

Nguyen et al, *TiG, 2018*