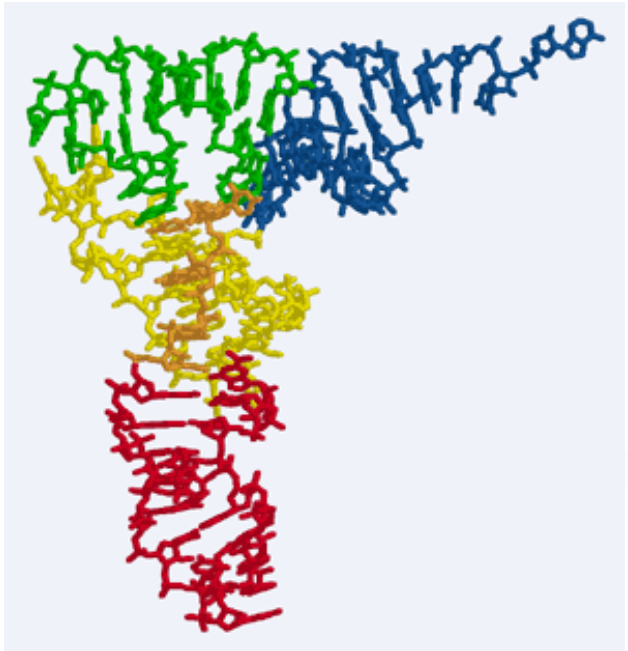
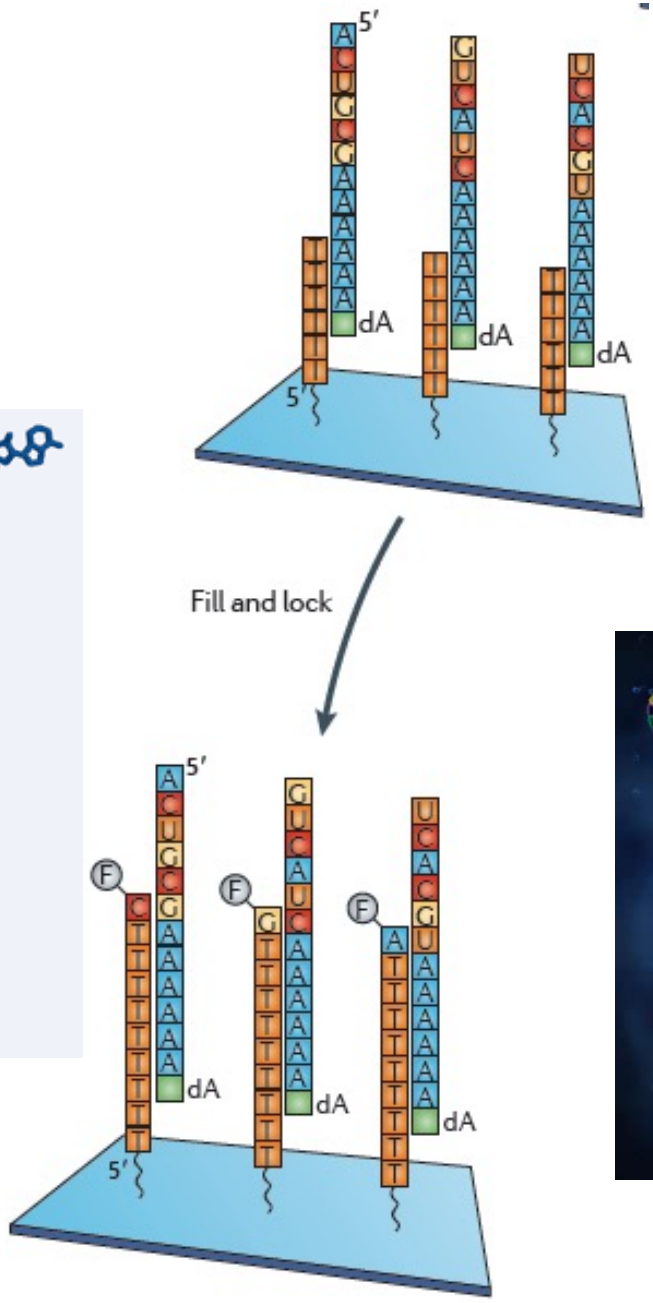
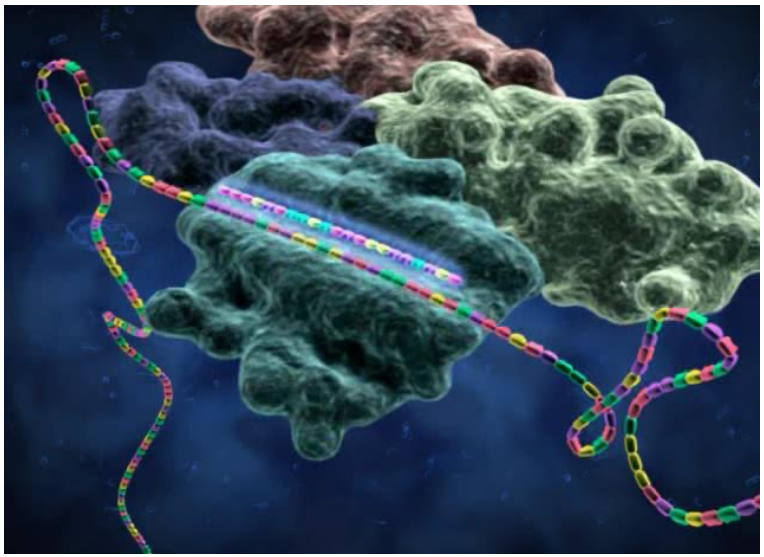
GLOBAL ANALYSES of RNAs and RNPs







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

- **tiling arrays**

microarrays with overlapping probes that cover the complete genome

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

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

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

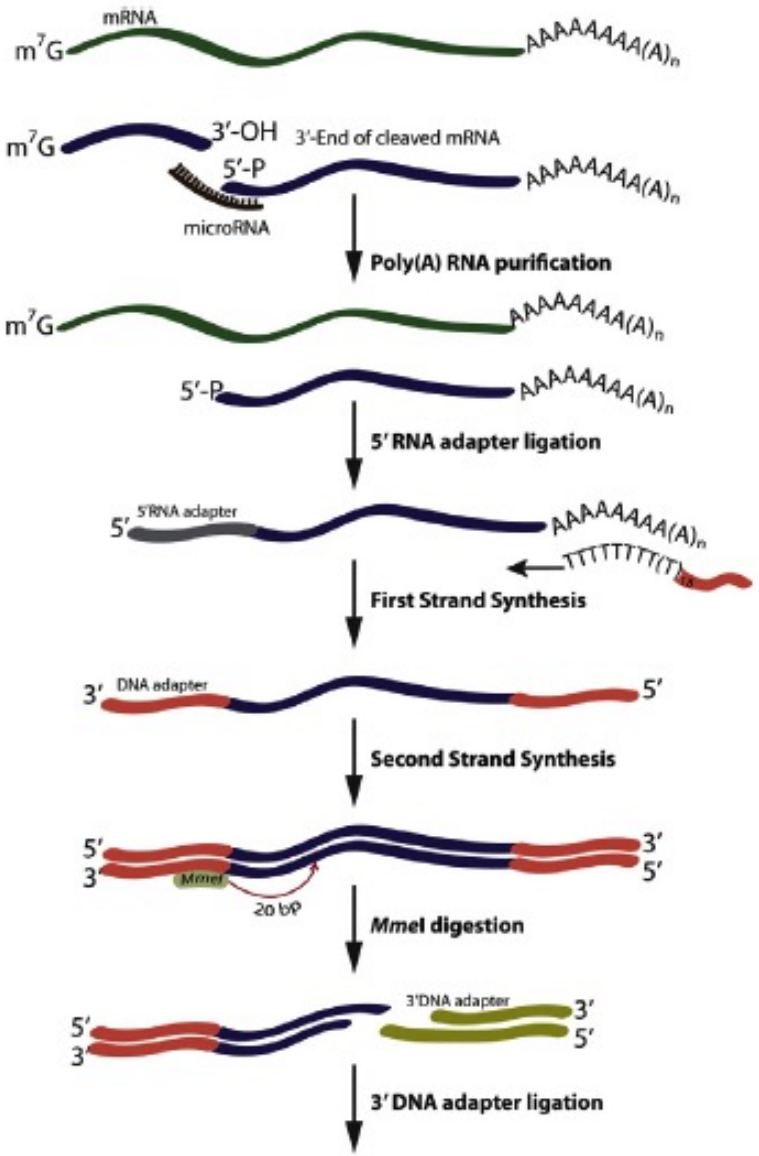
- **CRAC - CRosslinking and Analysis of cDNA**

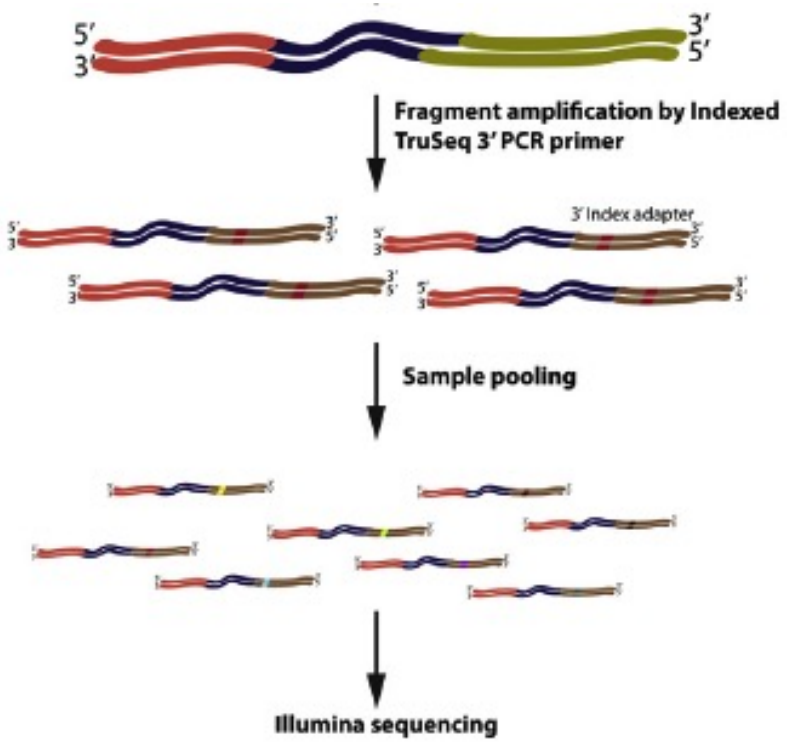
- **PAR-CLIP - PhotoActivatable ribonucleoside–enhanced CrossLinking and ImmunoPrecipitation**

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

PARE: Parallel Analysis of RNA End

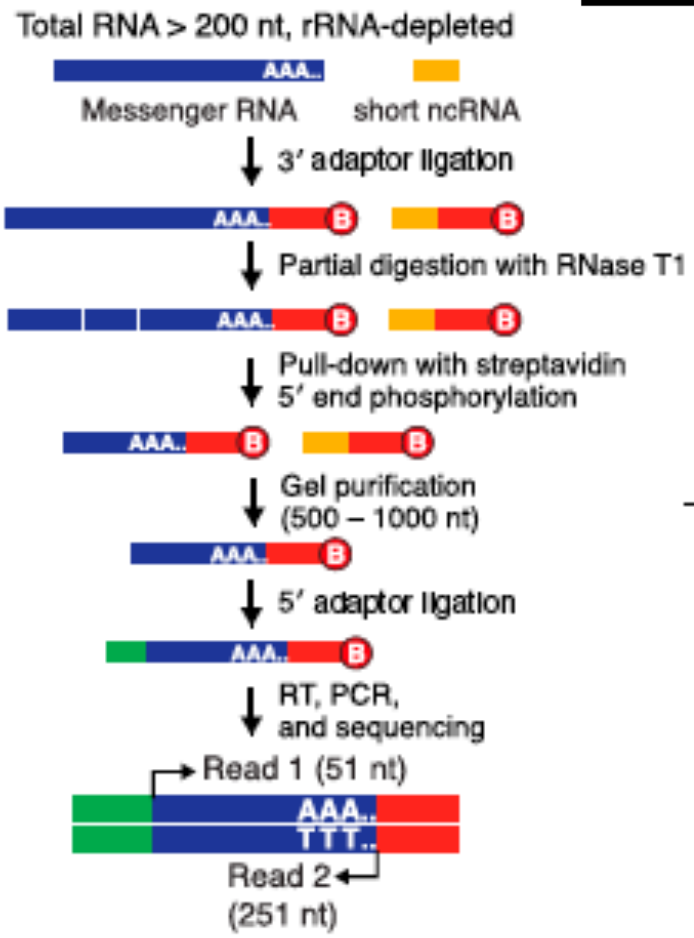
mRNA DEGRADOME RNA-seq

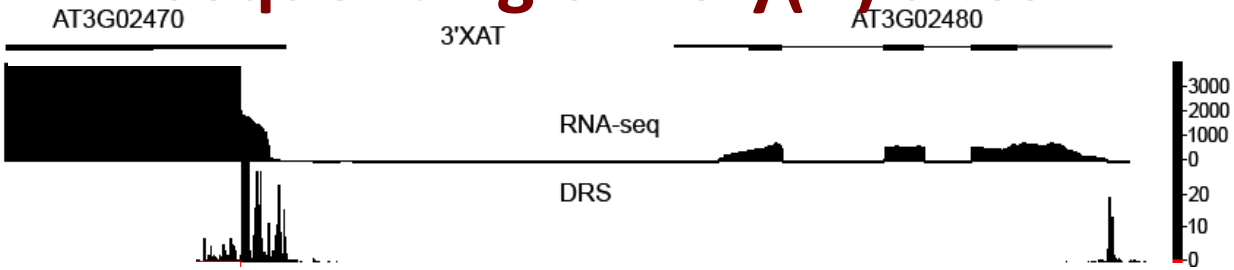




Zhai et al., Methods, 2014

DRS: Direct RNA sequencing of Poly(A) sites

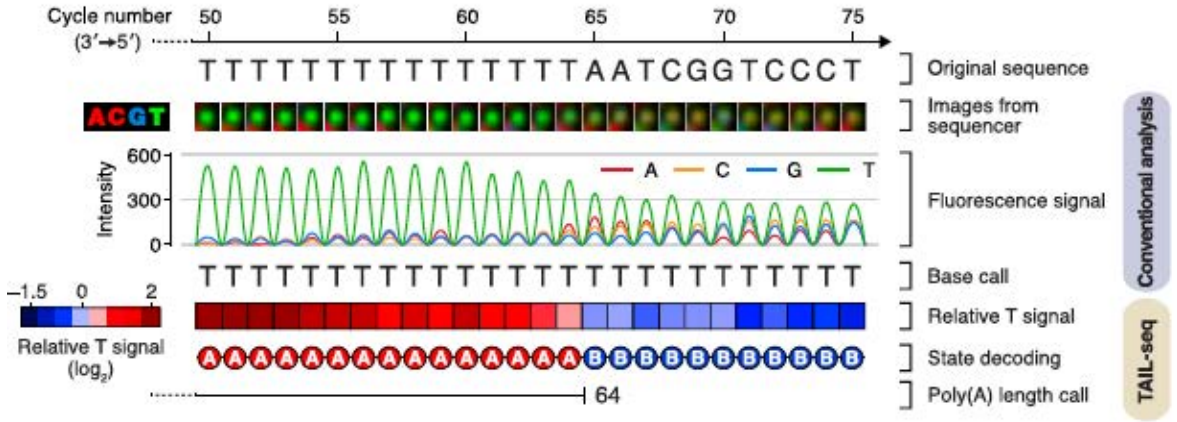




TAIL-seq: RNA 3’ end sequencing

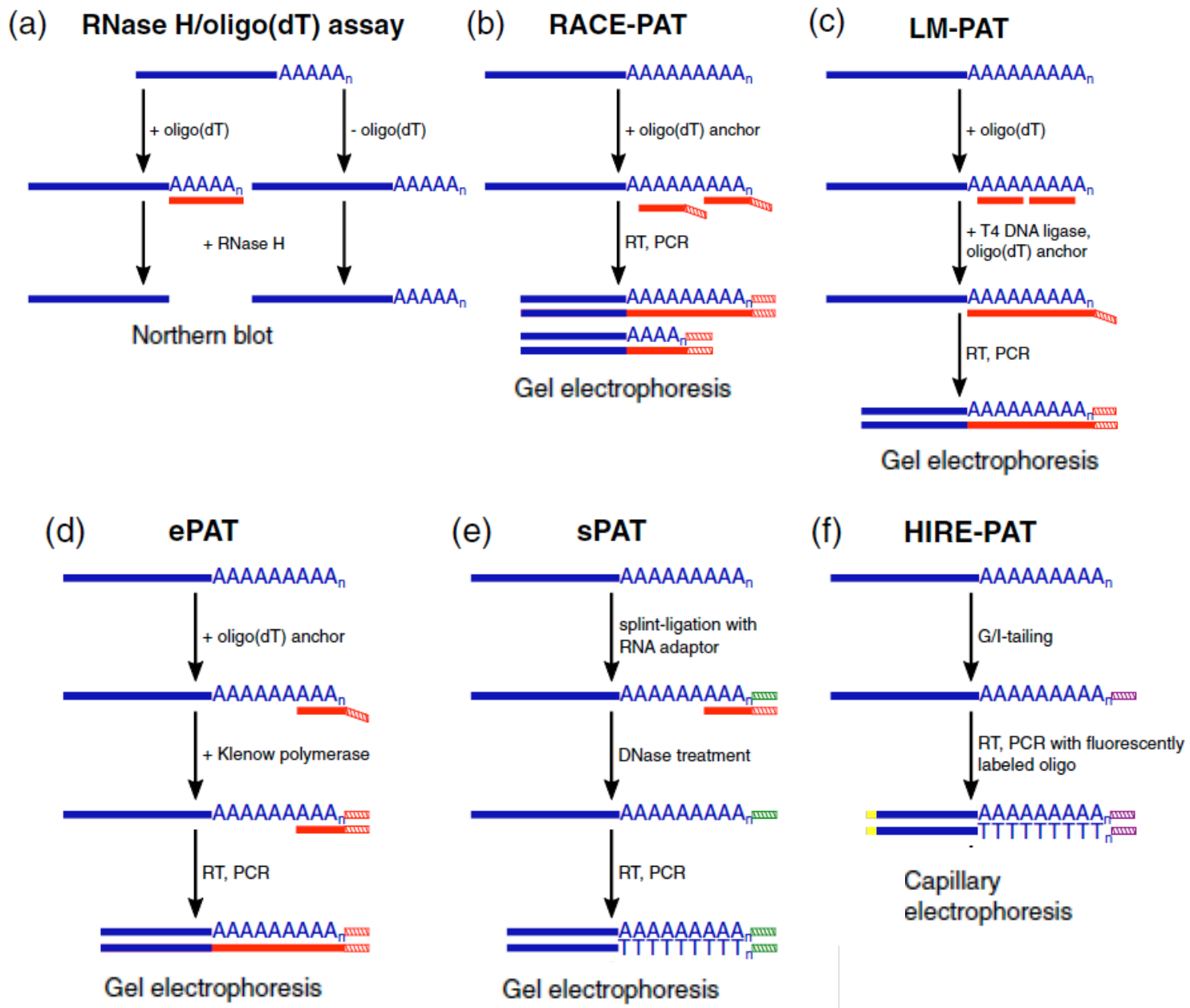
Poly(A) tail length and 3’ end modifications

(e.g. U-tailing)



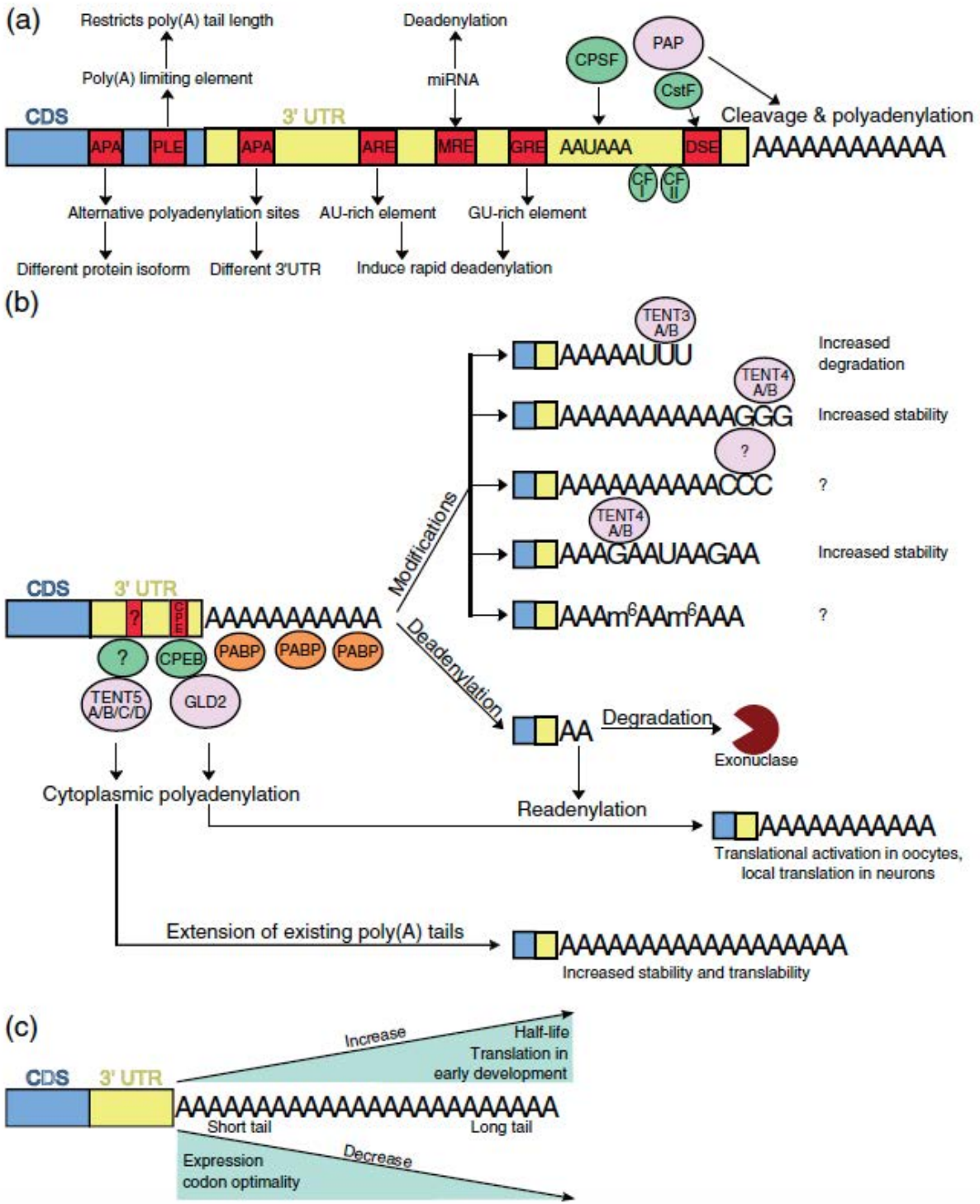
Sherstnev et al., Nat Str Mol Biol, 2012; Chang et al., Mol Cell, 2014

Poly(A) tail analyses, classical methods



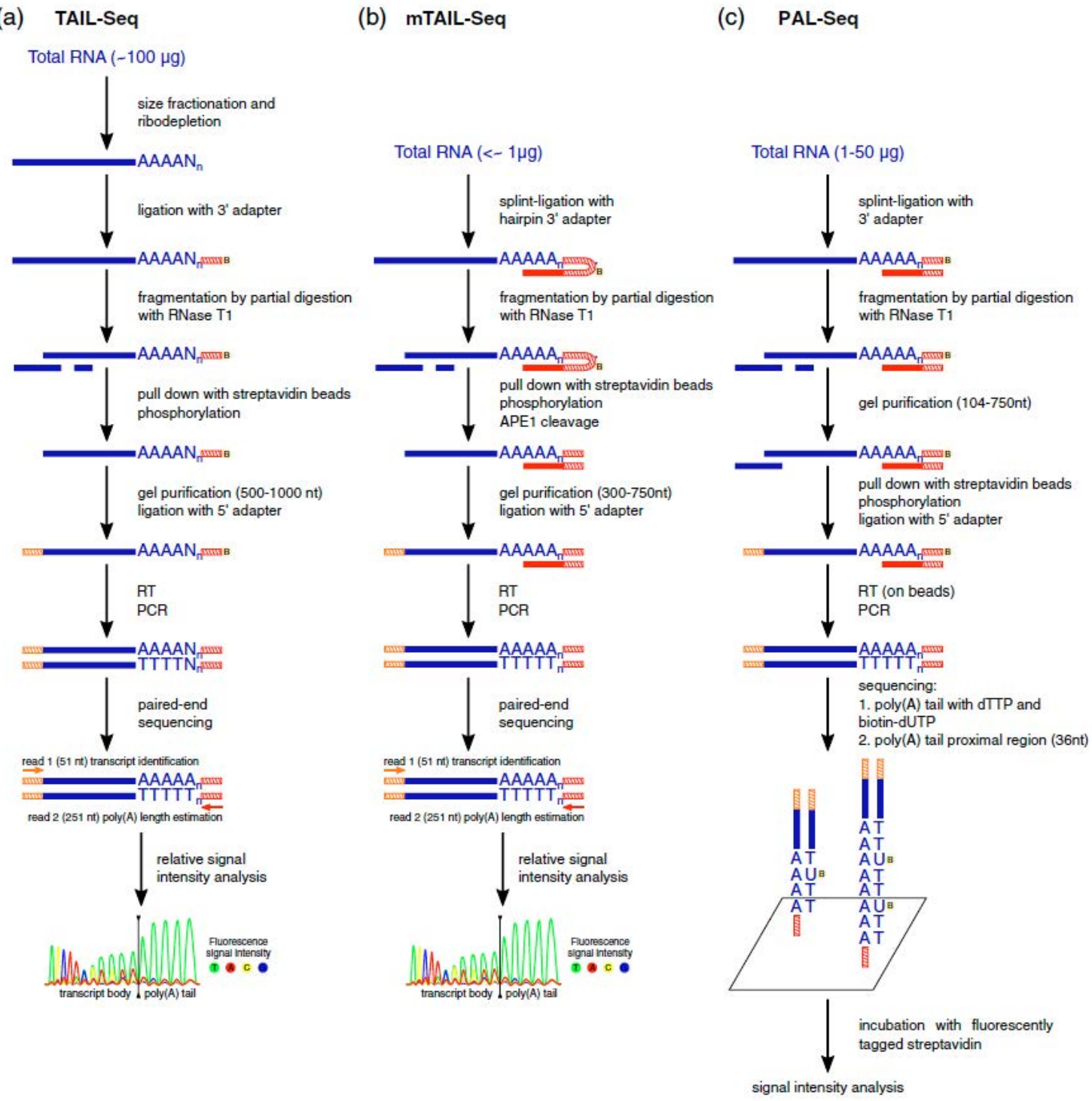
Brouze et al, WIREsRNA, 2022

3’ end and poly(A) tail analyses



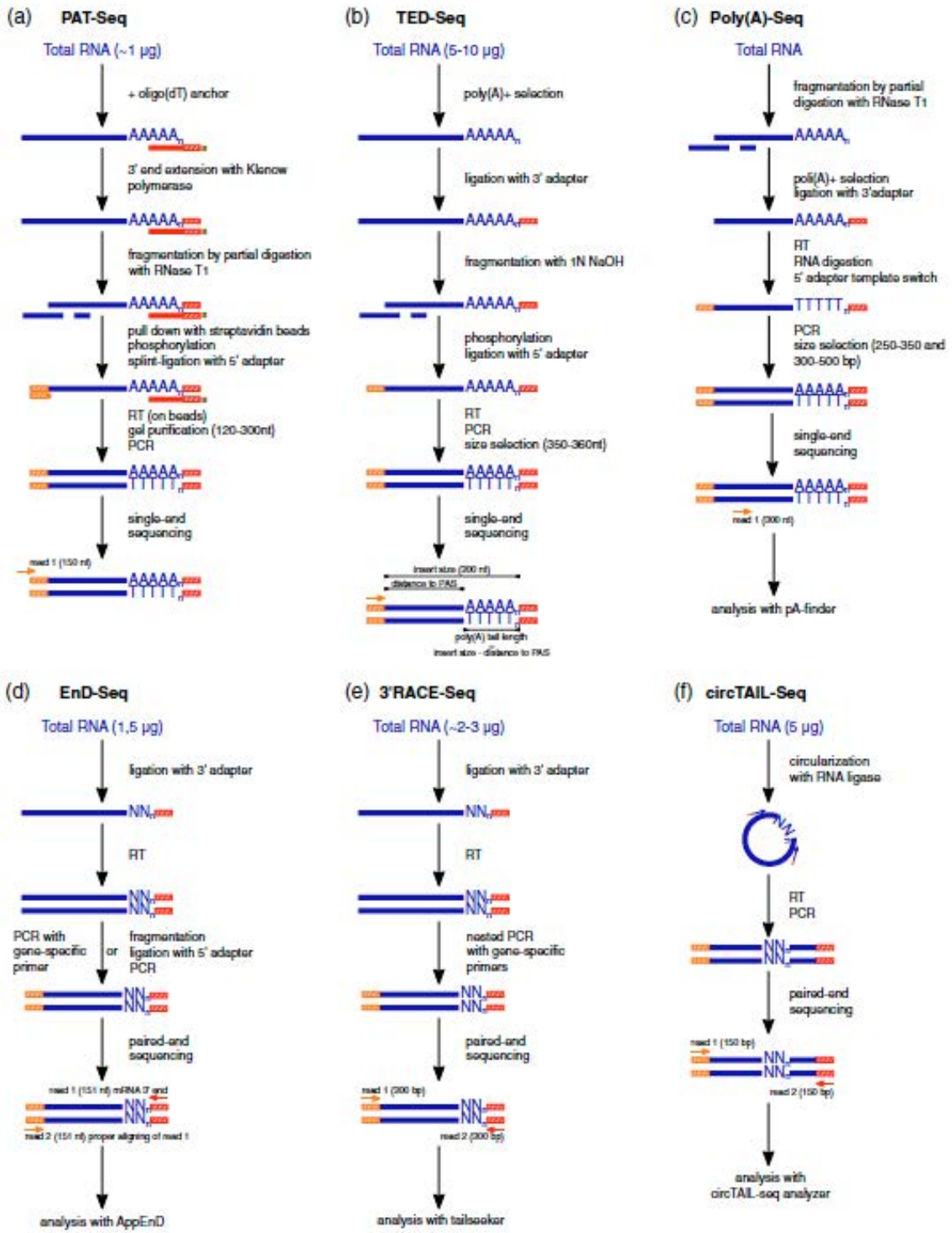
Brouze et al, WIREsRNA, 2022

Poly(A) tail analysis



Brouze et al, WIREsRNA, 2022

3’ end RNA analysis



Brouze et al, WIREsRNA, 2022

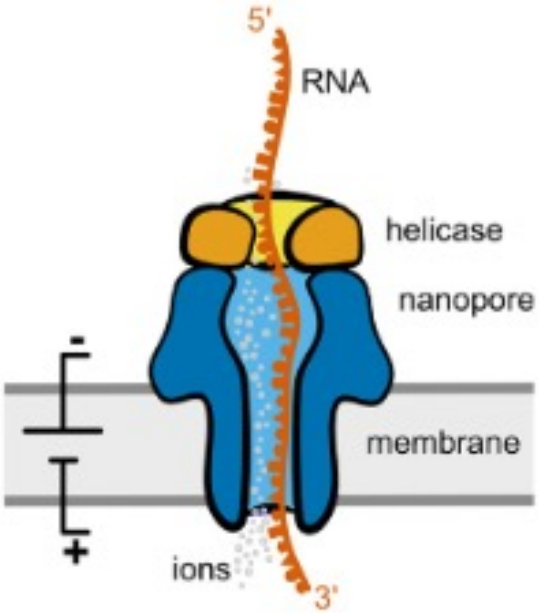
Nanopore long read sequencing

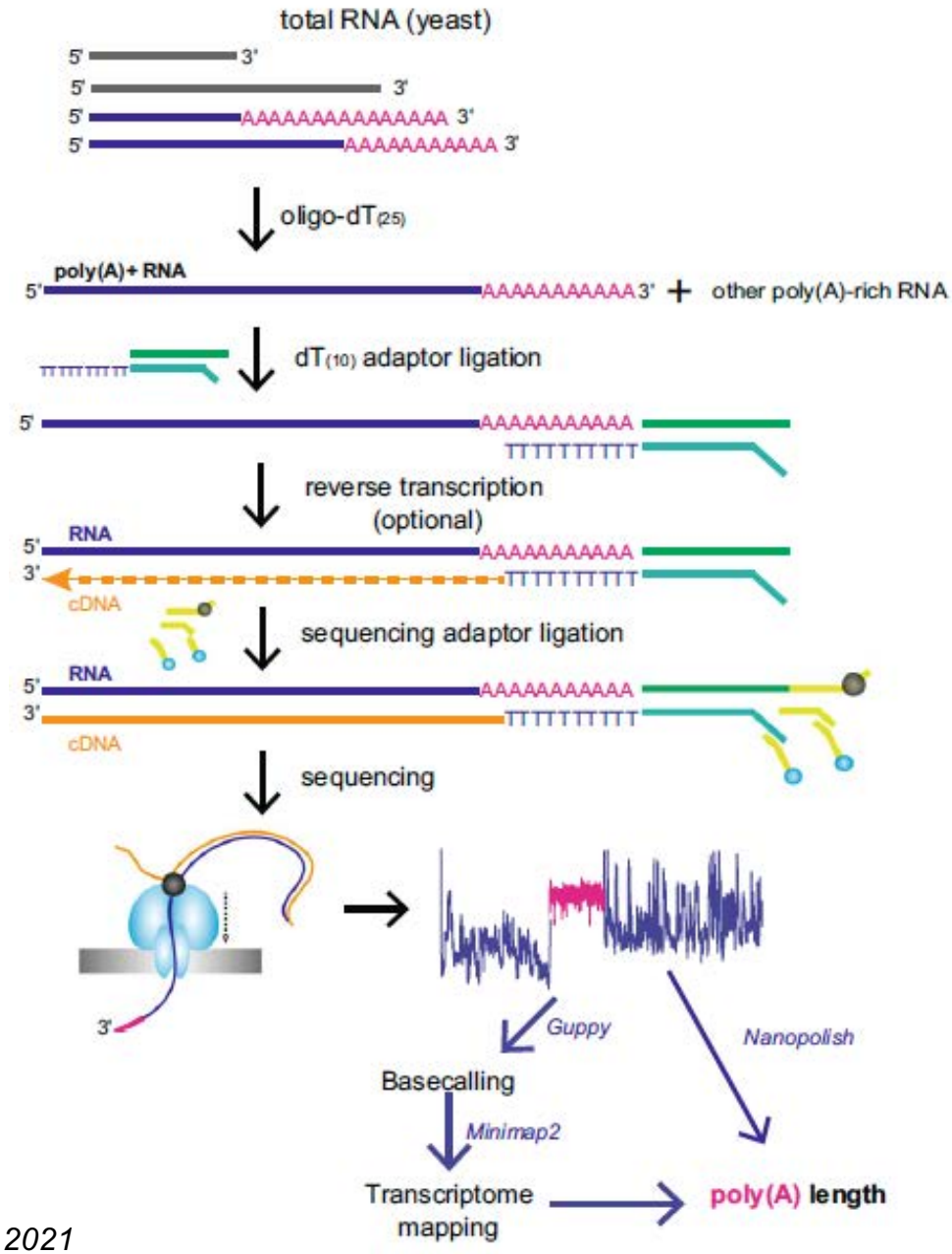
DNA and RNA-seq

DRS or cDNA-based

Poly(A) tail analysis

RNA modification mapping



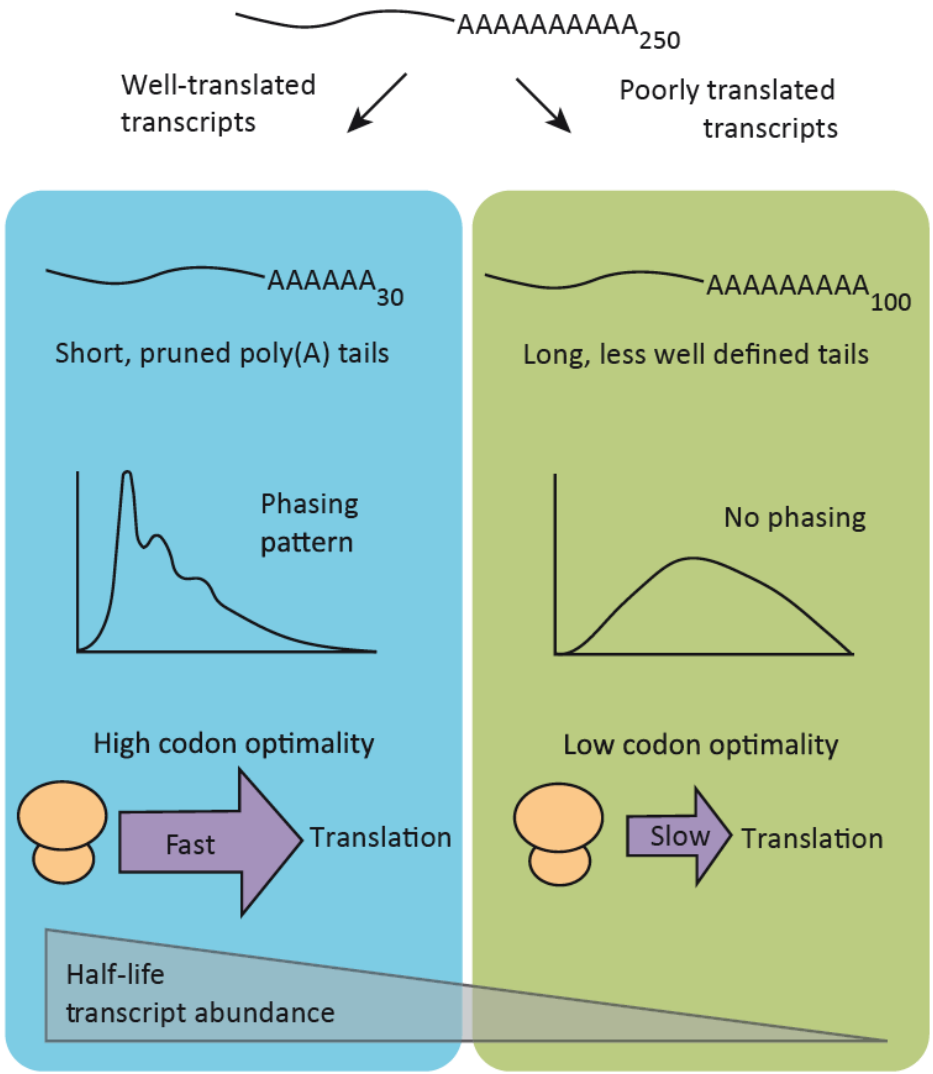


- For polyA$^+$ RNA
- For nonpolyadenylated RNA addition of poly(A) or poly(I) is required
- Lower deepth than NGS

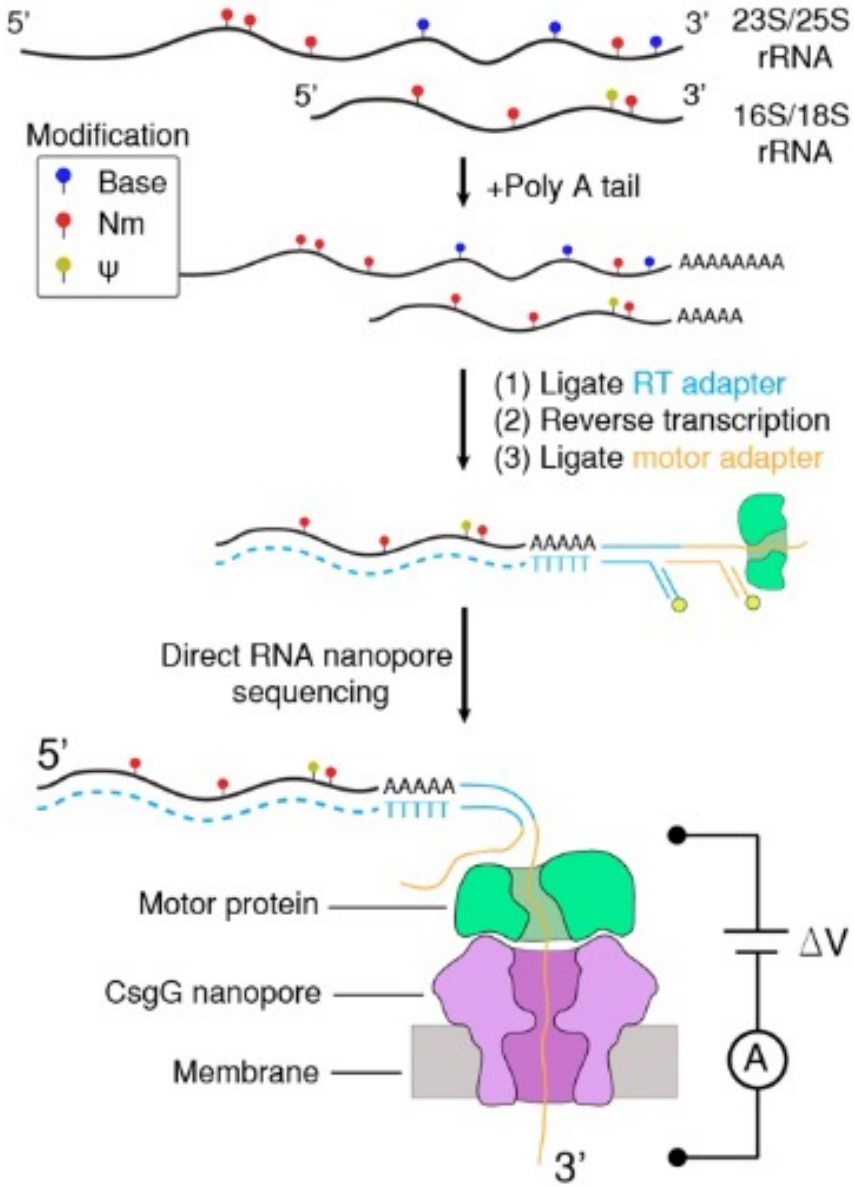
Ray and Hasselberth, Front Genet 2022; Tudek et al, Nat Comm, 2021

Nanopore

Poly(A) tail analyses



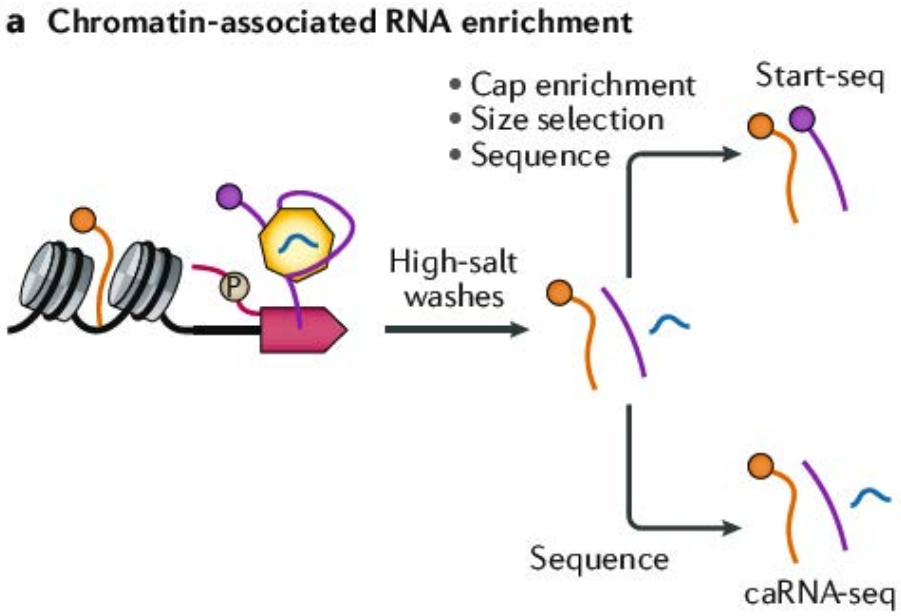
RNA modifications



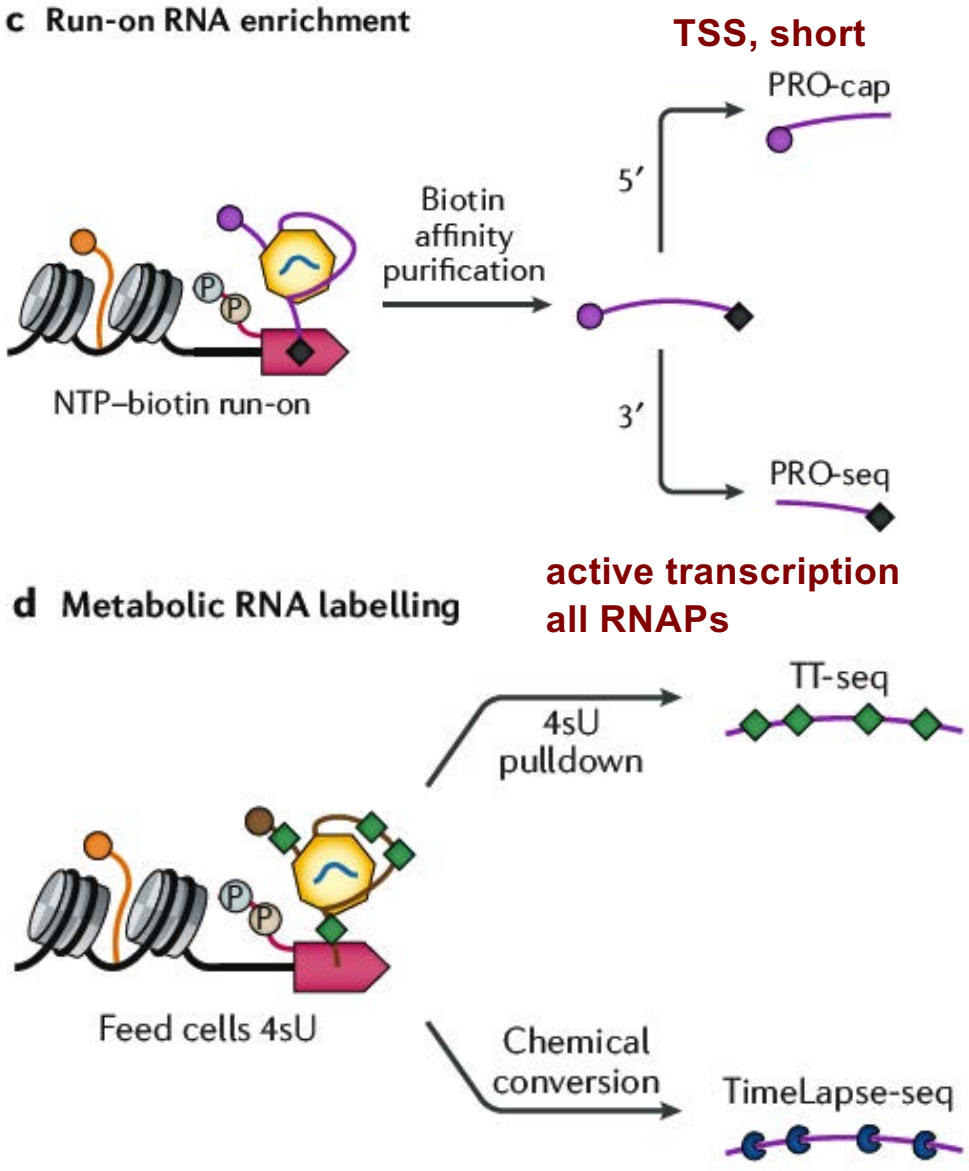
Nicholson and Pasquinelli TiCB 2018

Nascent RNA analyses

IP-based, formaldehyde crosslink

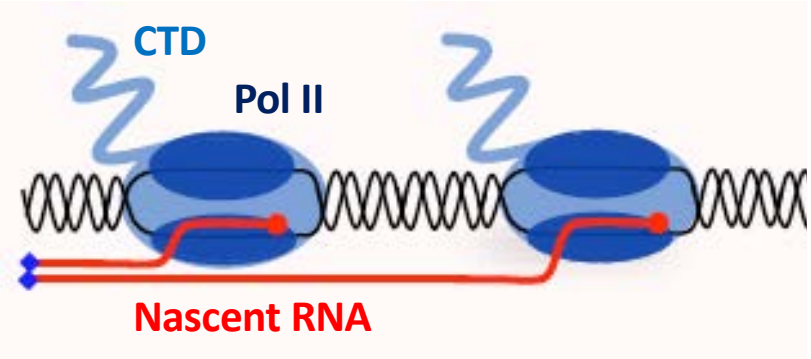


Purification of transcribed RNAs



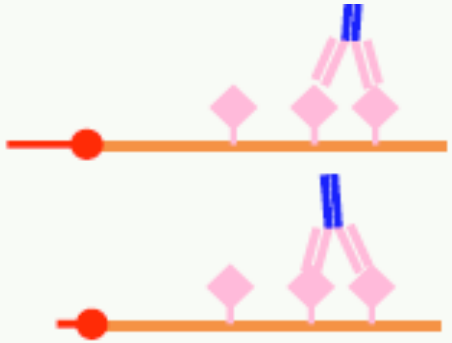
Wissink et al, Nat Rev Genet, 2019

Nascent RNA analyses



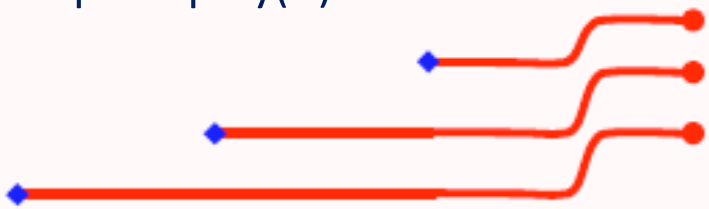
NET-seq
Nuclear GRO-seq

Label nascent RNA with BrUTP/4sUTP
IP with α-BrU or
Convert 4sU to biotin
Isolate biotinylated RNA



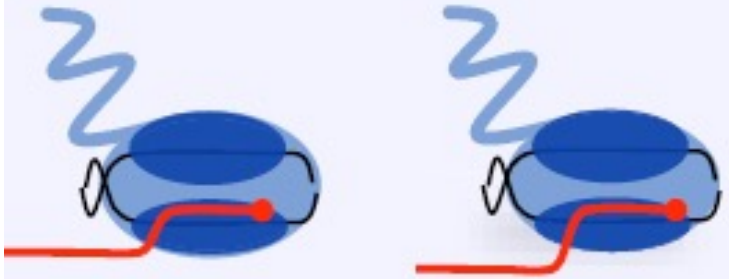
ChaRNA-seq

Prepare chromatin
Isolate chromatin-bound RNA
Deplete poly(A) and rRNA

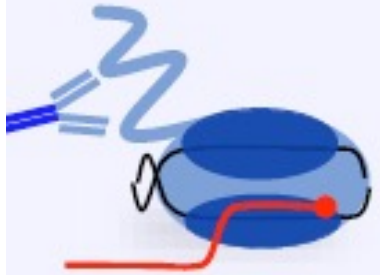


NET-seq

Treat with MNase
Release Pol II complex



IP with α-Pol II



Liu et al, Plant Cell, 2023

Nascent RNA methods

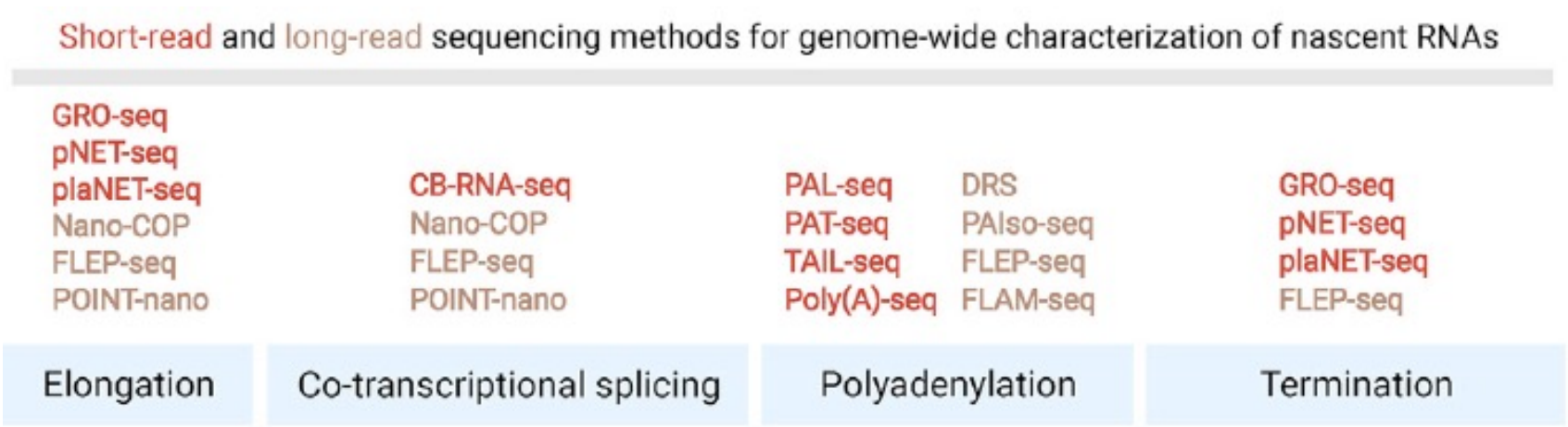
caRNA- seq chromatin-associated RNAseq
CoPRO coordinated precision run-on and sequencing
FISH fluorescence in situ hybridization
mNET-seq mammalian native elongating transcript seq
NET-seq native elongating transcript seq
PRO-cap precision run- on with cap selection
PRO-seq precision run- on seq
SL AM-seq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

Wissink et al, Nat Rev Genet, 2019

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

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]

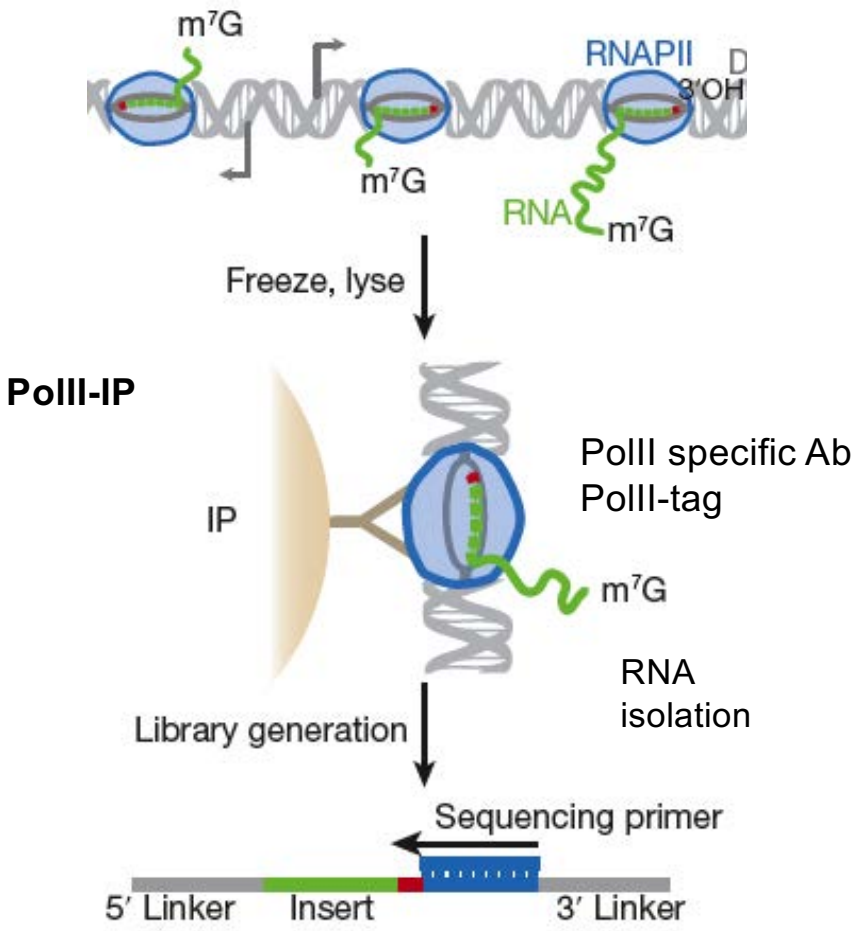


Wissink et al, Nat Rev Genet, 2019
Quin et al, Curr Op Plant Biol 2022

Analysis of Nascent Transcripts

NET-seq

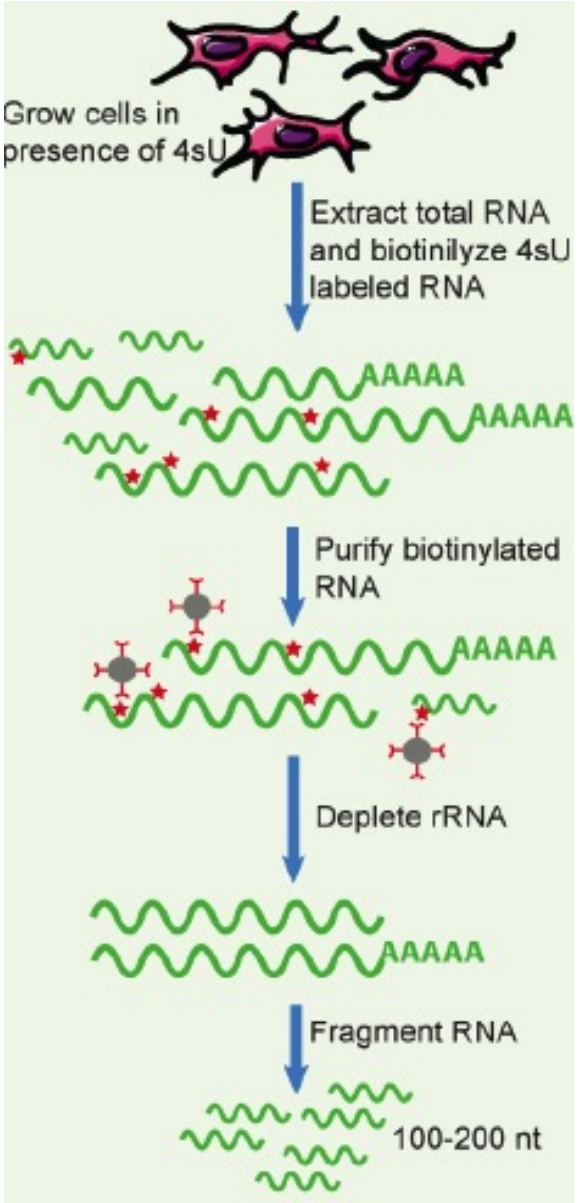
I. Isolation of PolII-bound RNAs



Churchman and Weissman, Nature, 2011

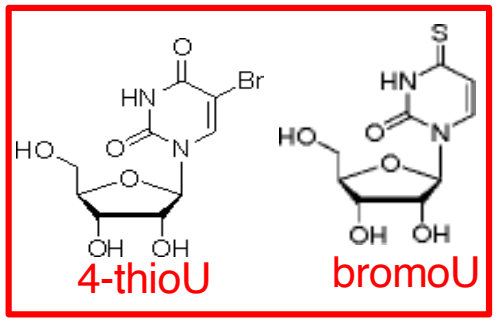
GRO-seq

II. Nascent RNA labeling with 4sU



Spicuglia et al., Methods, 2013

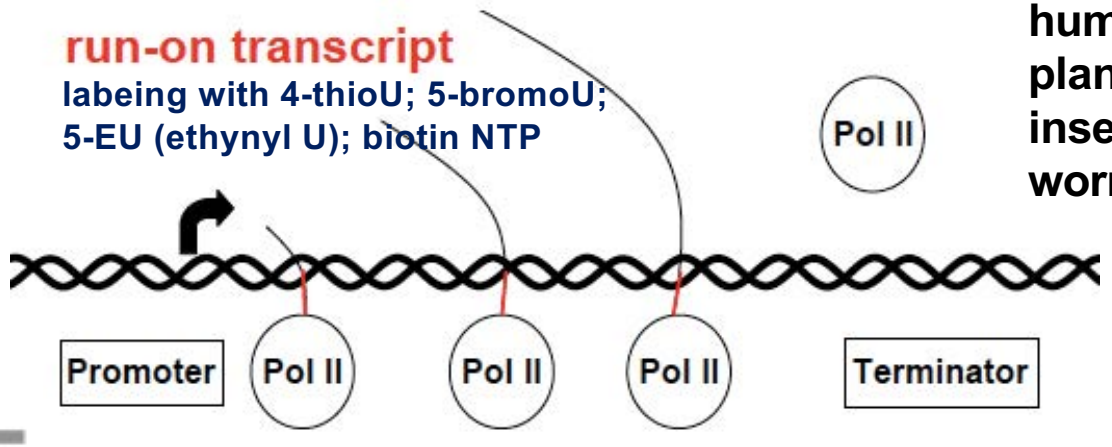
Analysis of Nascent Transcripts: GRO-seq

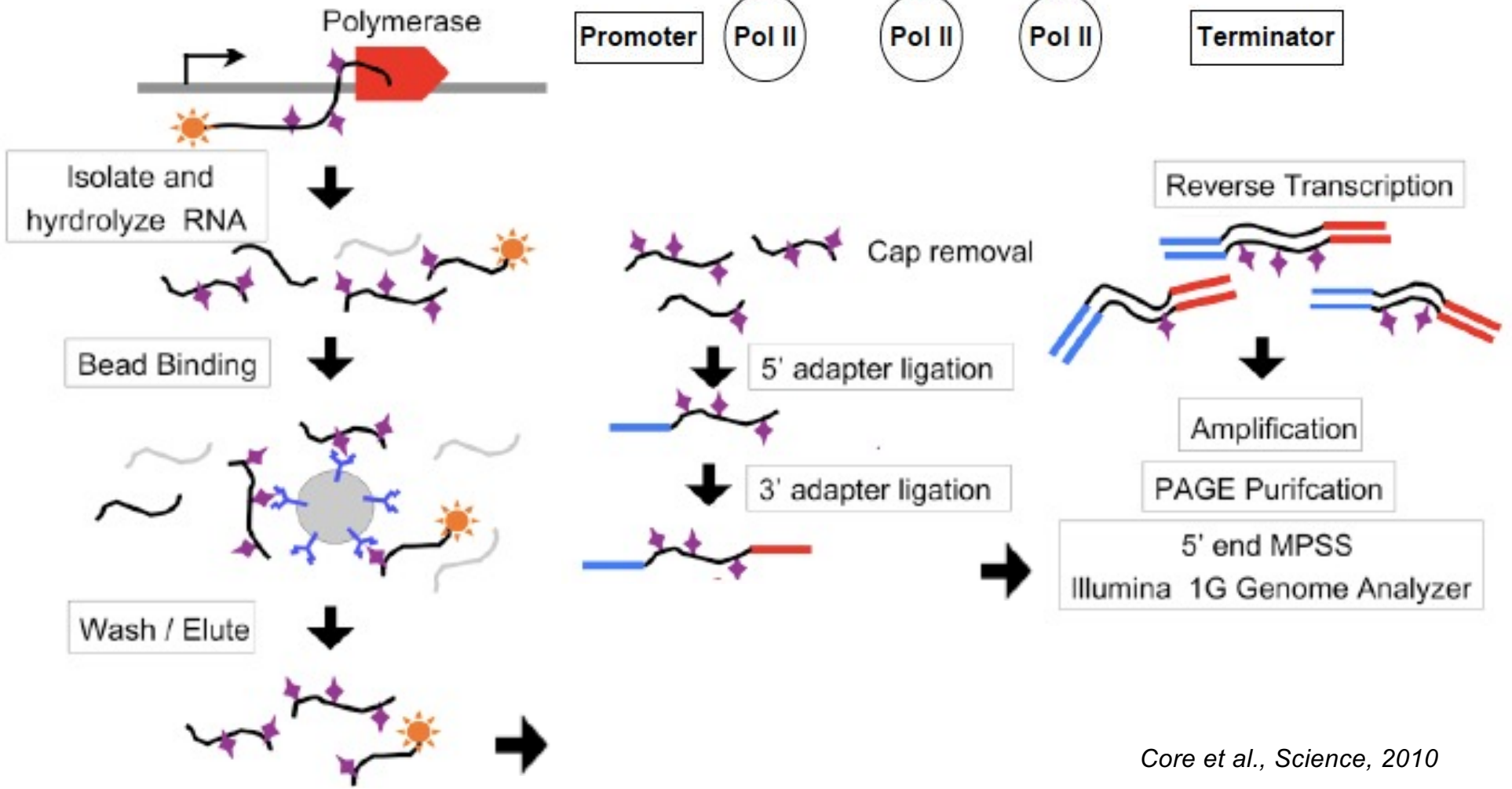


run-on transcript

labeing with 4-thioU; 5-bromoU; 5-EU (ethynyl U); biotin NTP

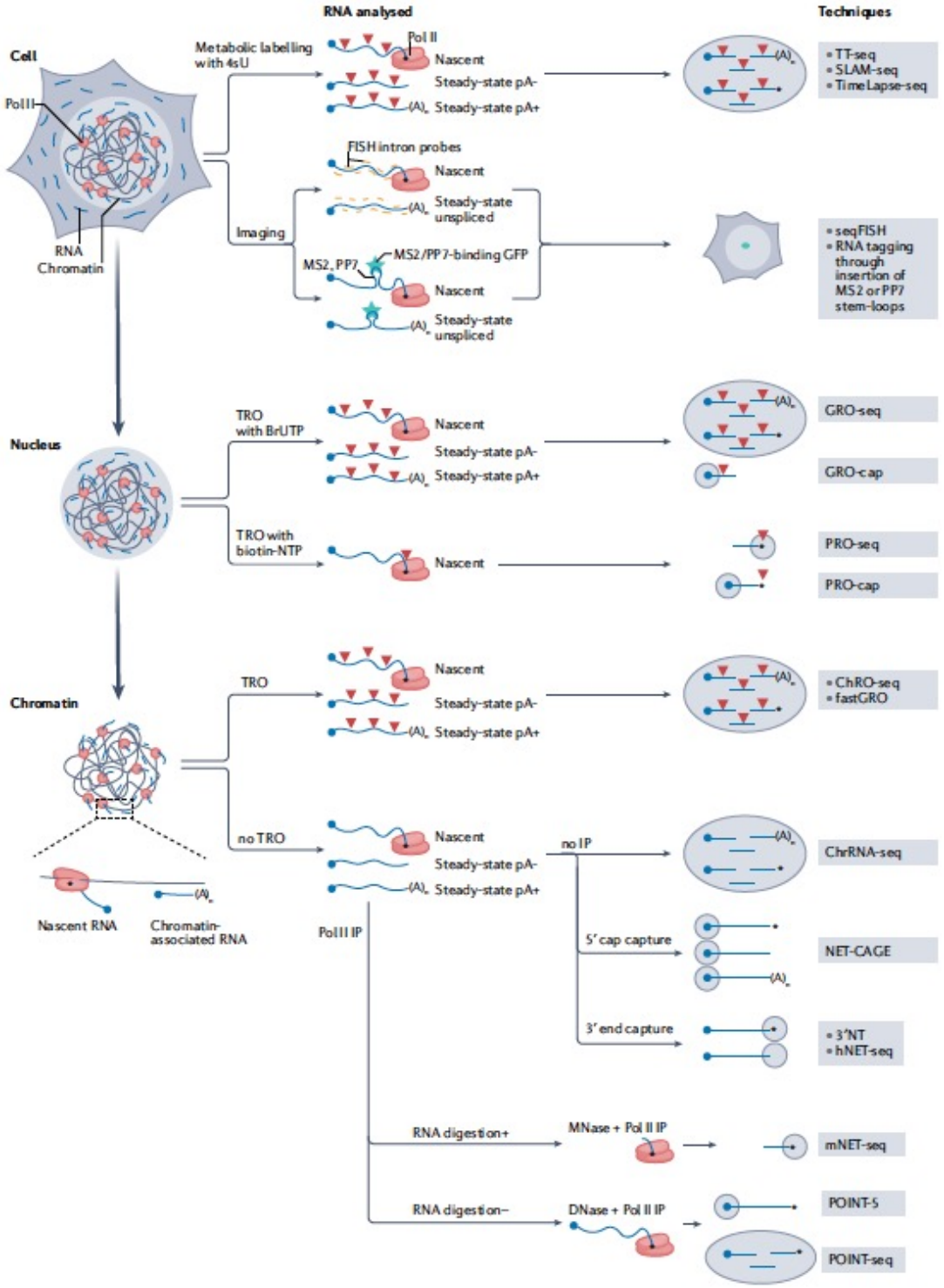
human
plant
insect
worm





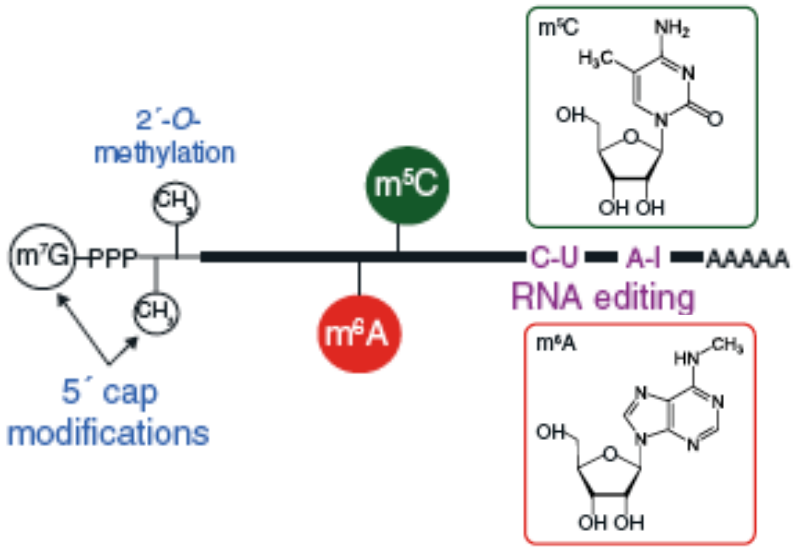
Core et al., Science, 2010

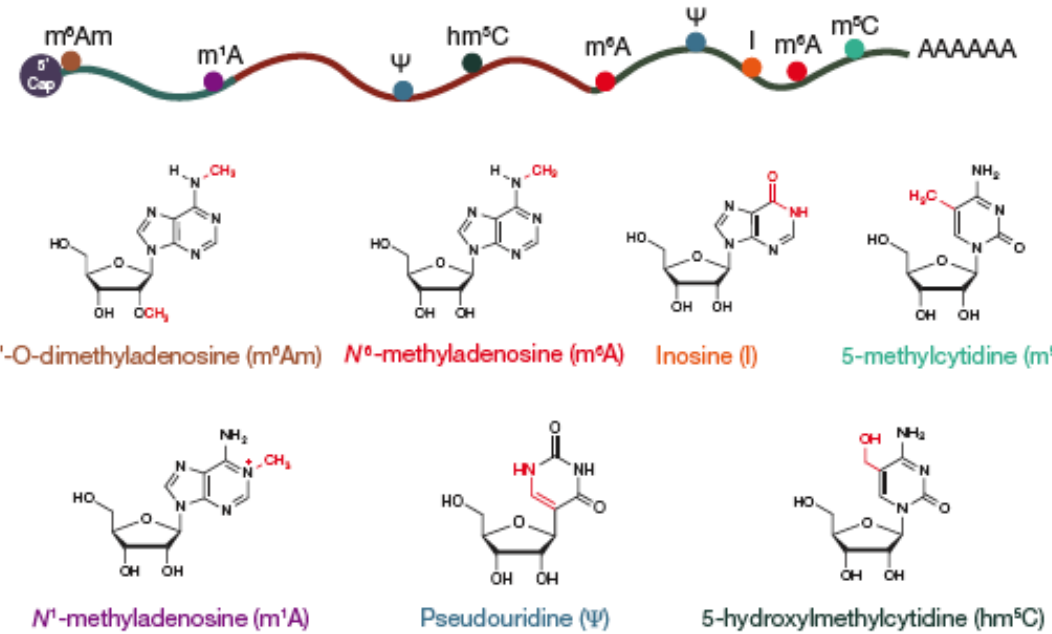
Nascent RNA analysis in mammalian cells



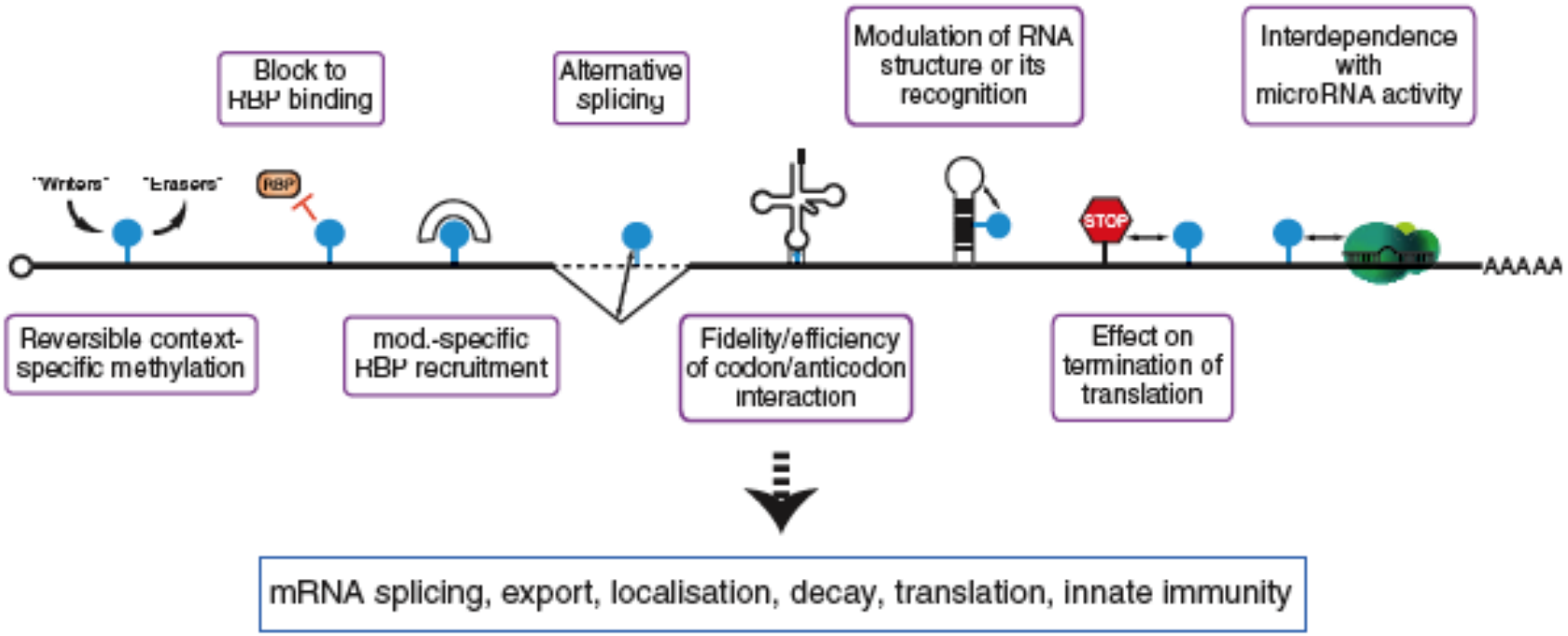
Nojima and Proudfoot, Nat Rev Mol Cell Biol, 2022

RNA modifications



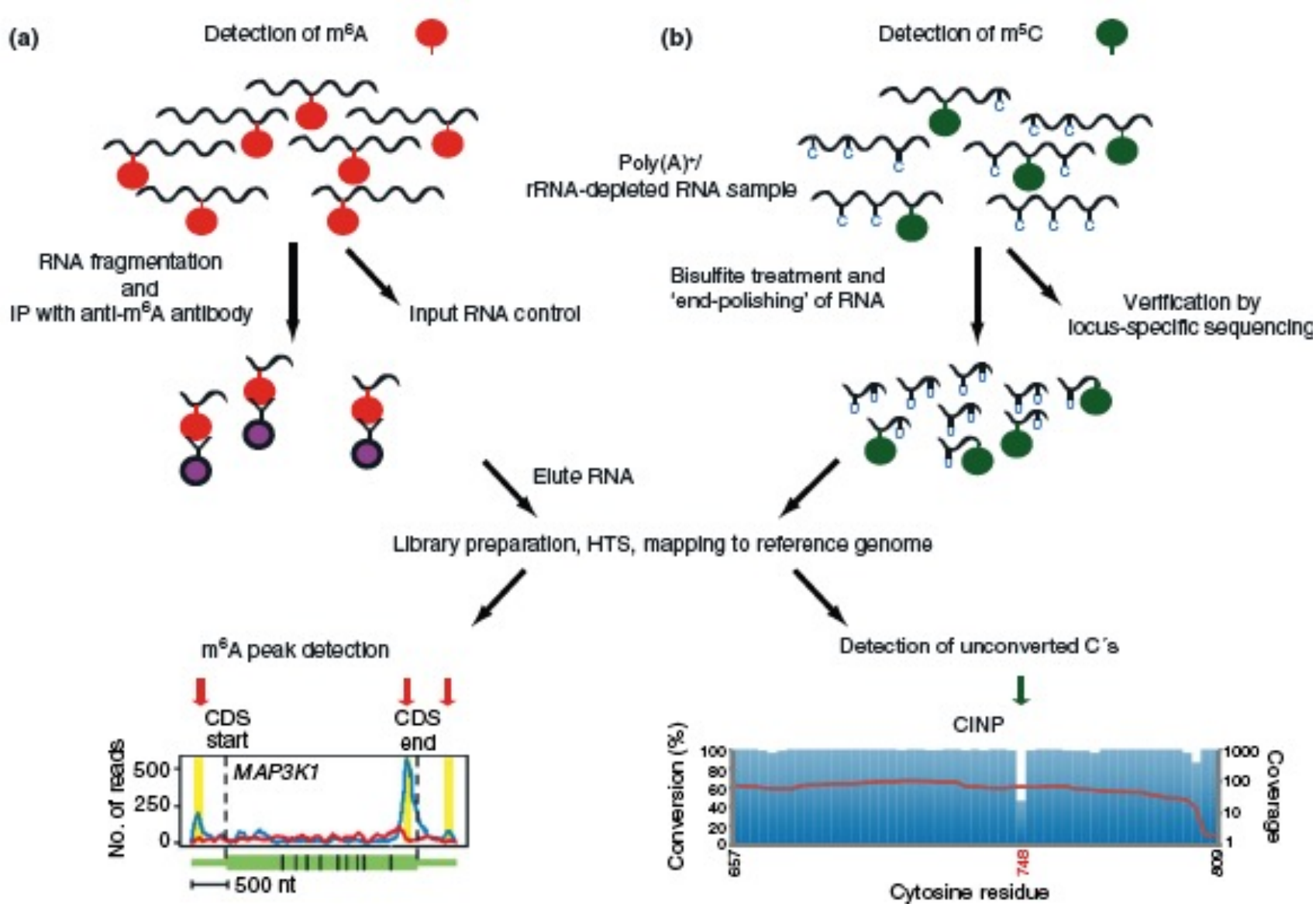


FUNCTIONS



Sibbrit et al, WIREsRNA 2013

RNA modifications



Sibbrrit et al, WIREsRNA 2013

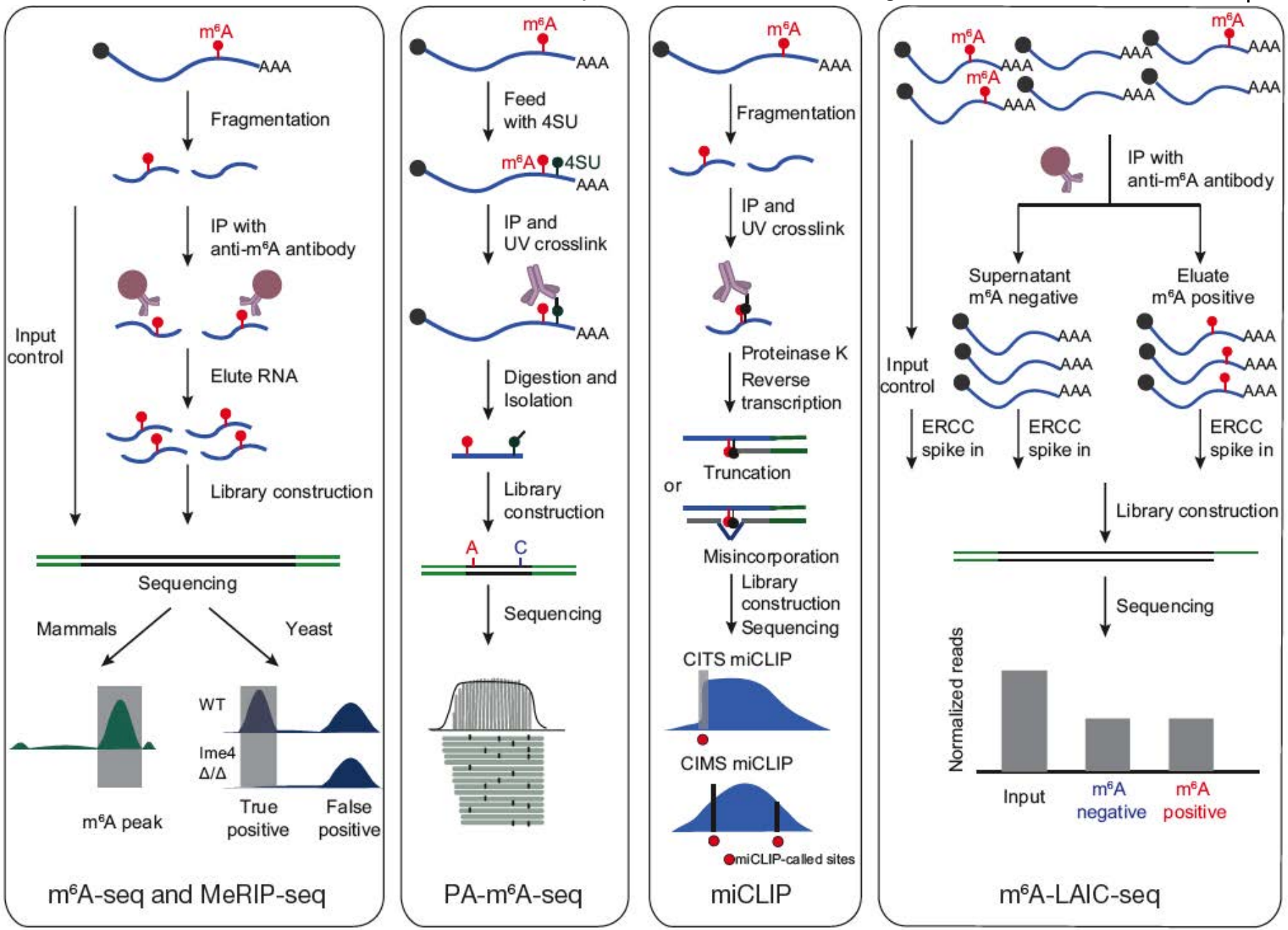
m⁶A RNA-seq

m6A-specific Ab IP seq

photo-crosslinking assisted m6A seq

m6A individual-nucleotide resolution crosslinking & IP

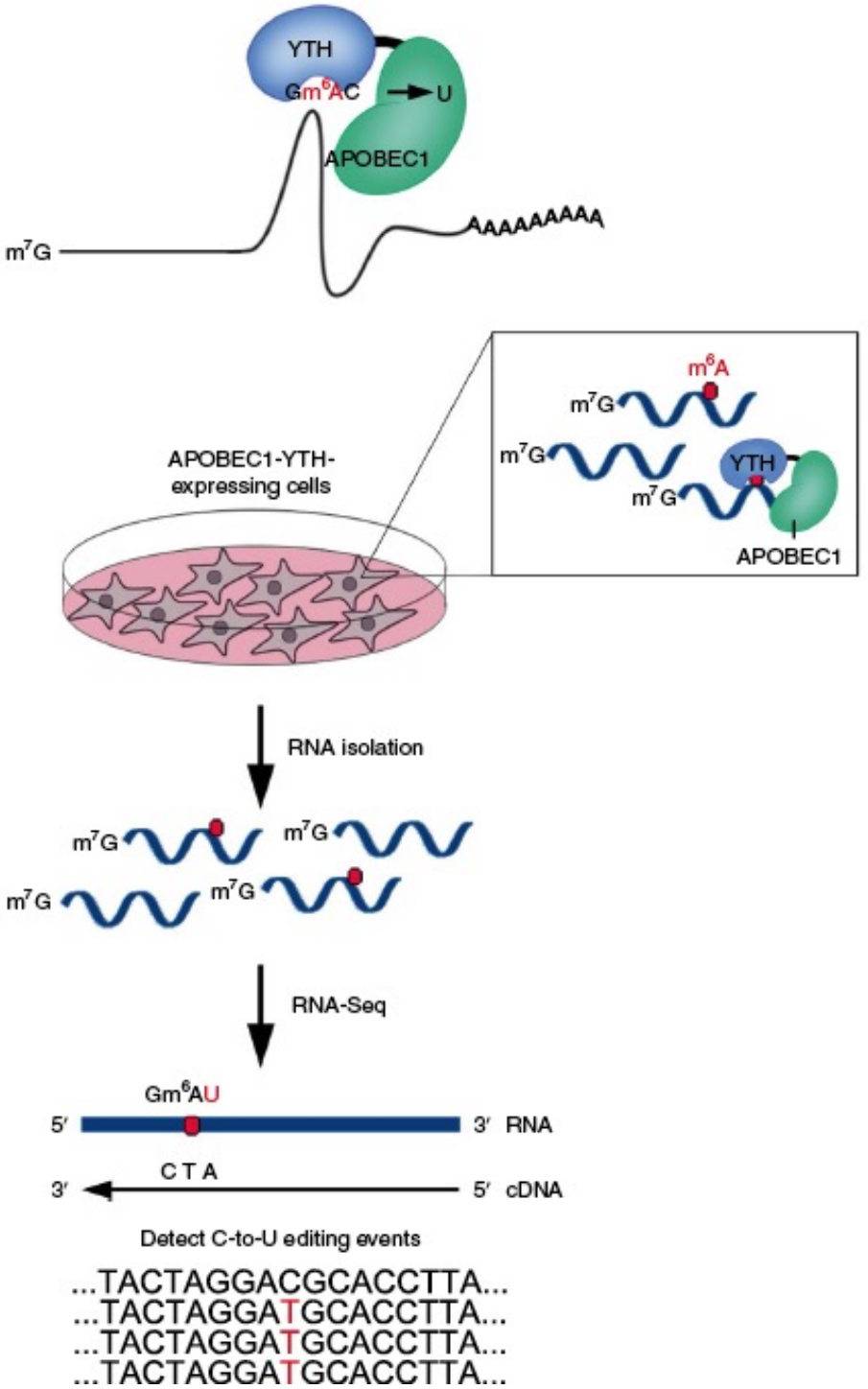
m6A-level and isoform-characterization seq



Li at al, Nat Methods, 2017

antibody-free m6A-seq DART-seq

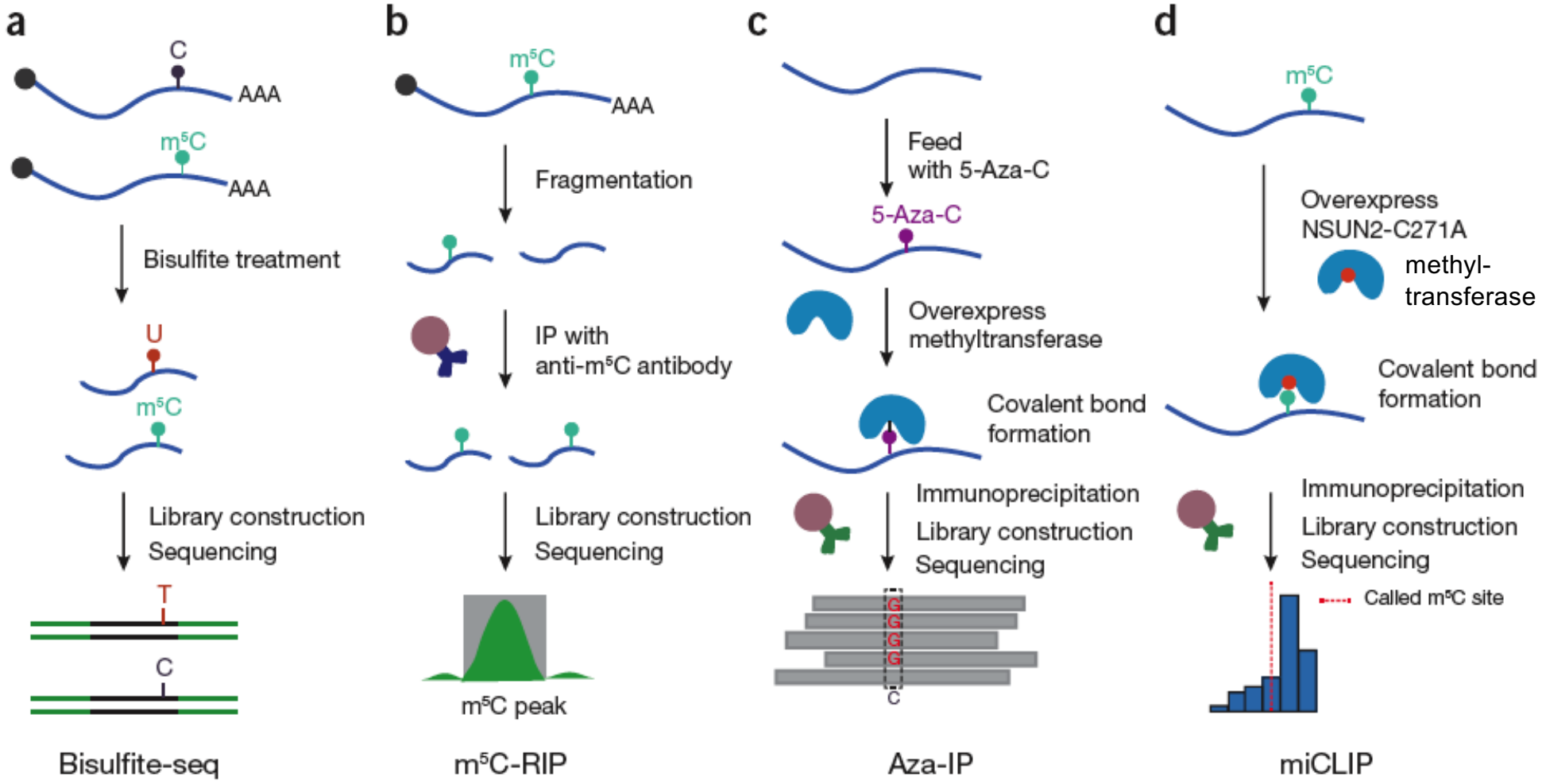
deamination adjacent to RNA modification targets



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

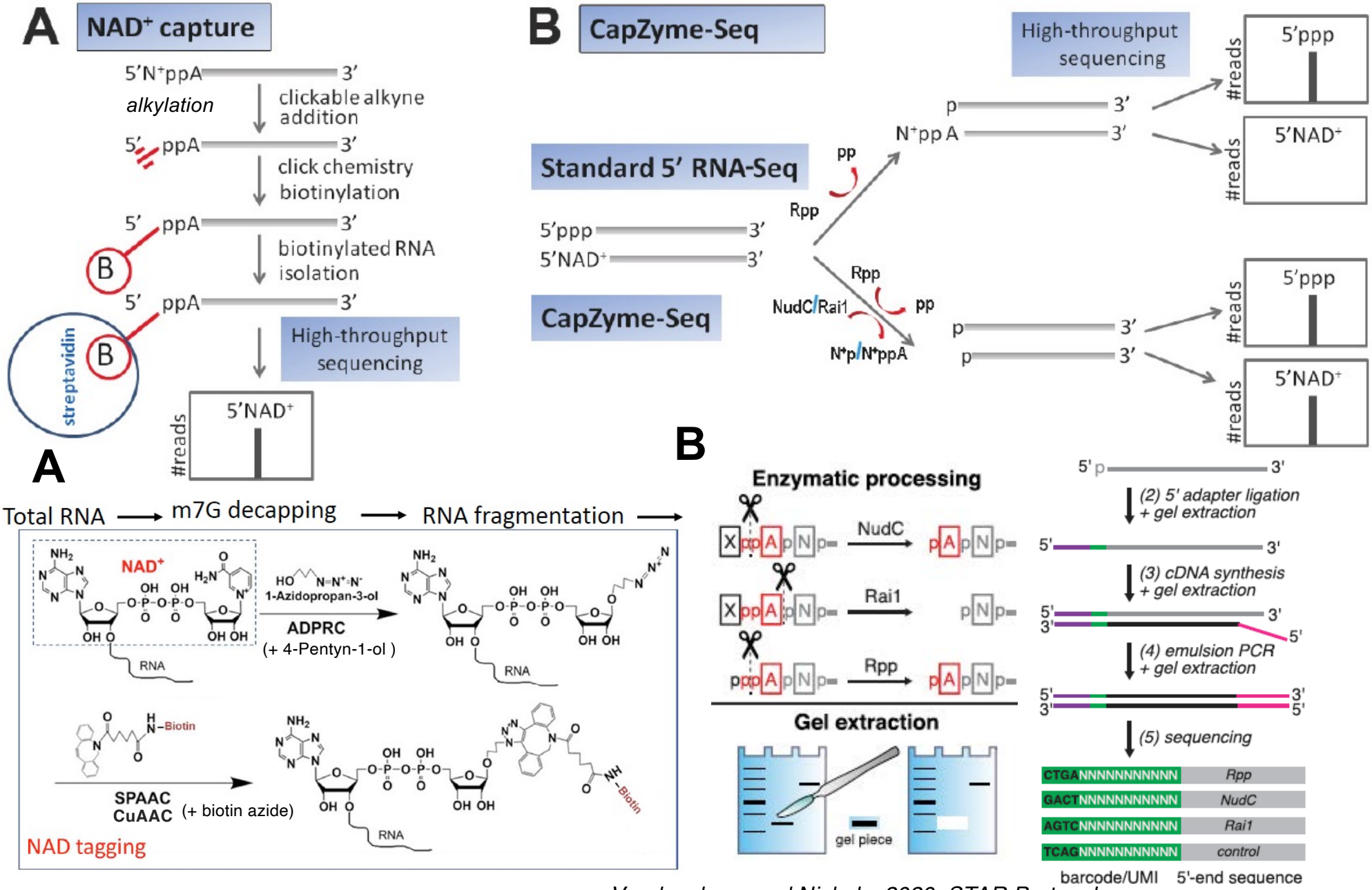
Meyer, Nature Methods 2019

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:

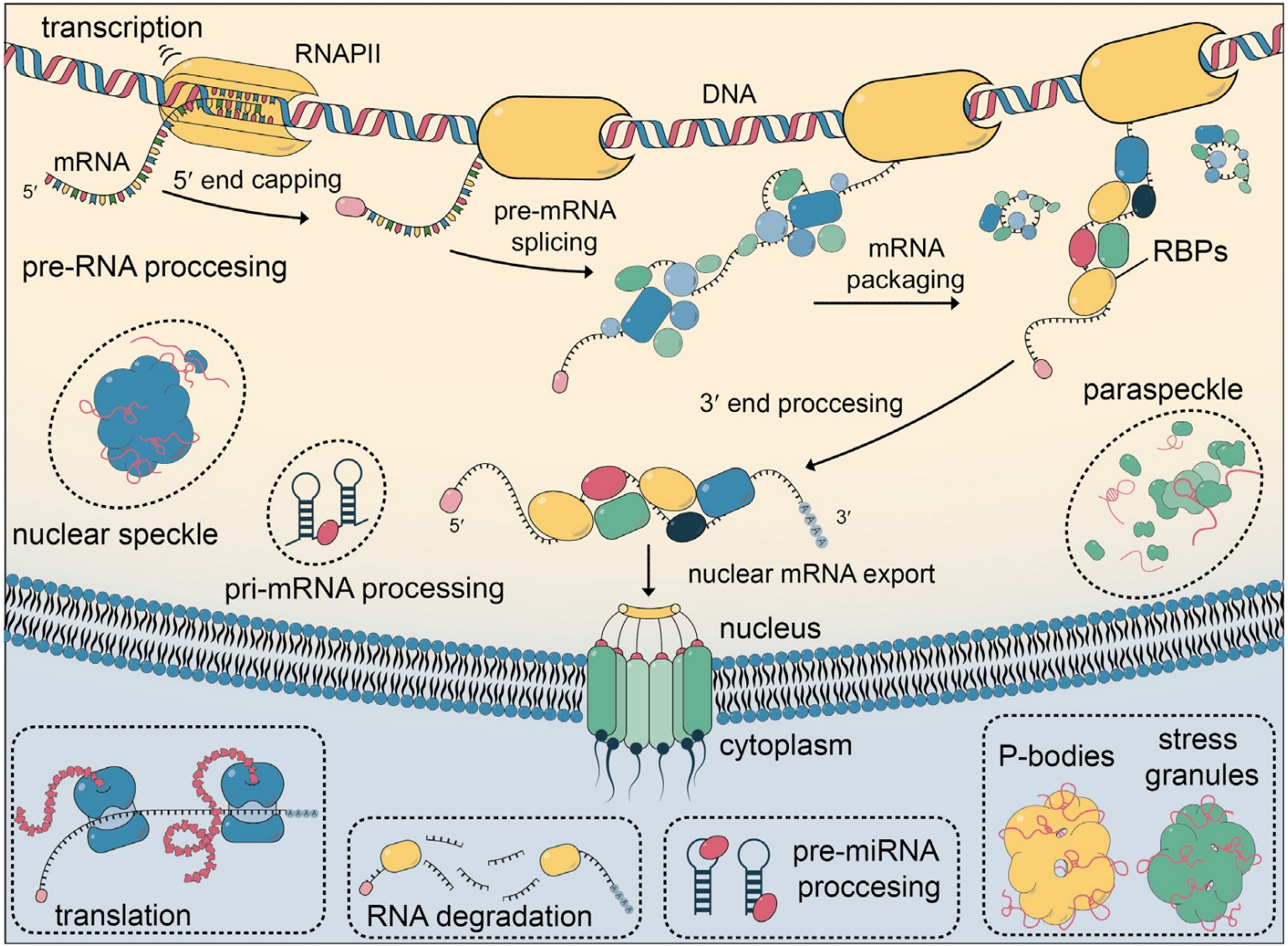
RNA-proteins

RNA-DNA

RNA-RNA

RNA structure

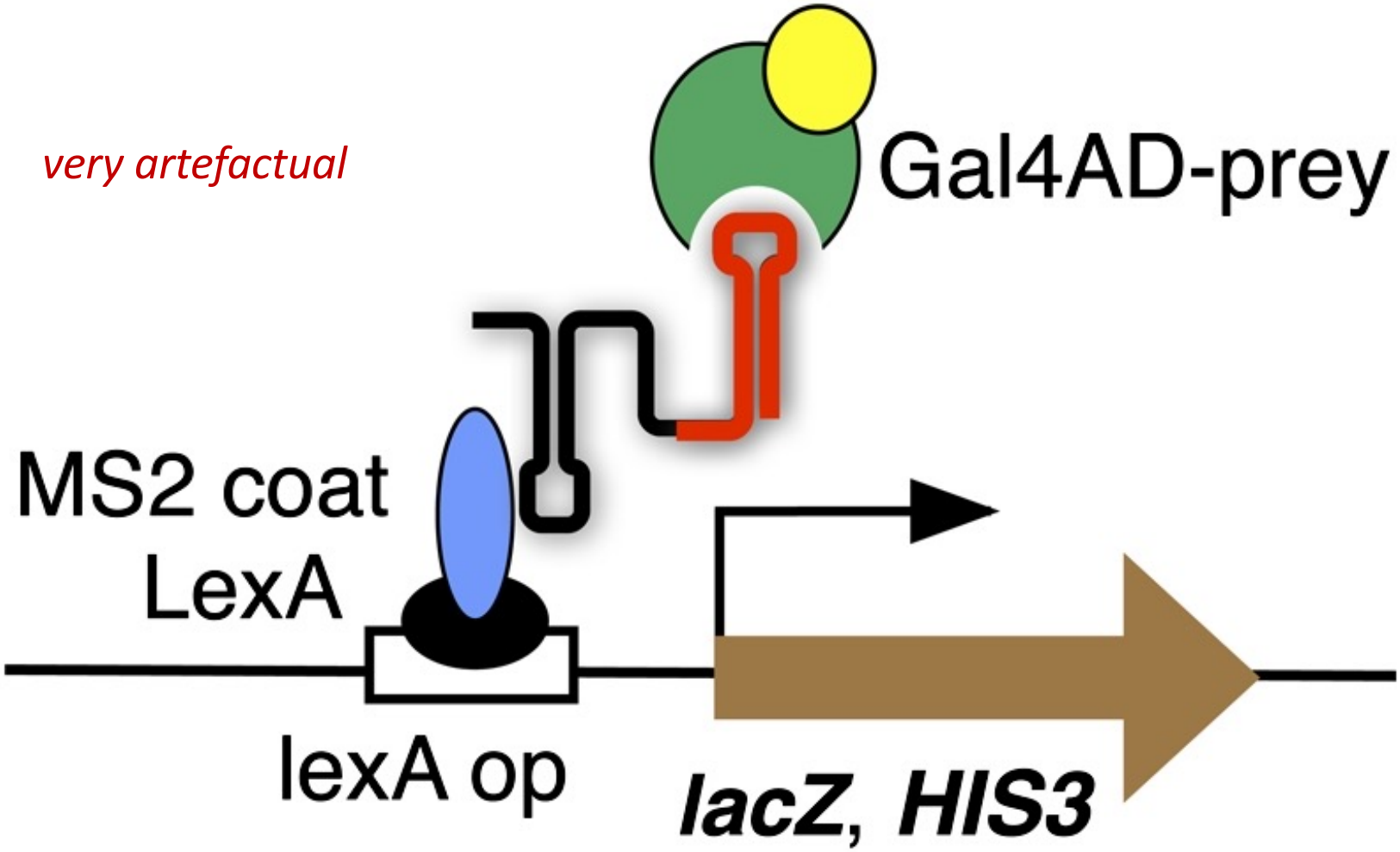
RBP - RNA binding proteins



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

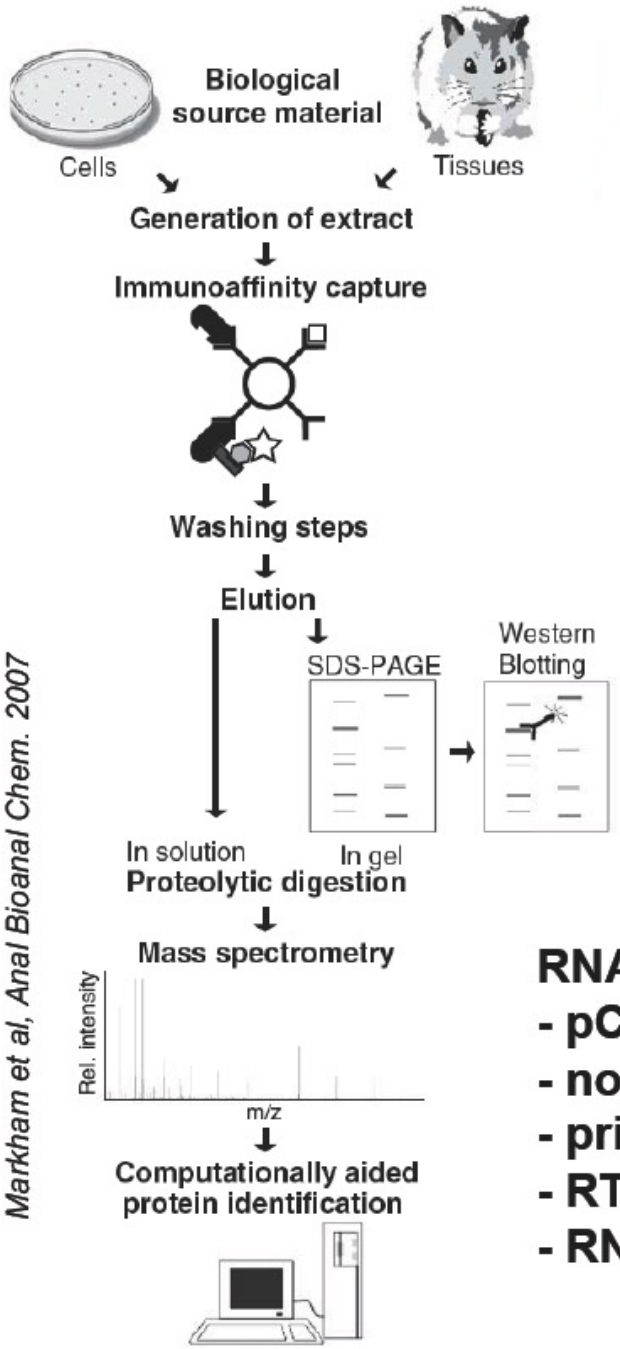
Genetic Screen- Yeast Three Hybrid



RNA insert is expressed in the context of **RNA** vector sequences tethered upstream of ***lacZ*** and ***HIS3*** reporter genes via a **MS2** coat–**LexA** fusion protein. Gene activation depends on binding of the **Gal4** activation domain–**prey** fusion protein.

RNP Immunoprecipitation (IP)

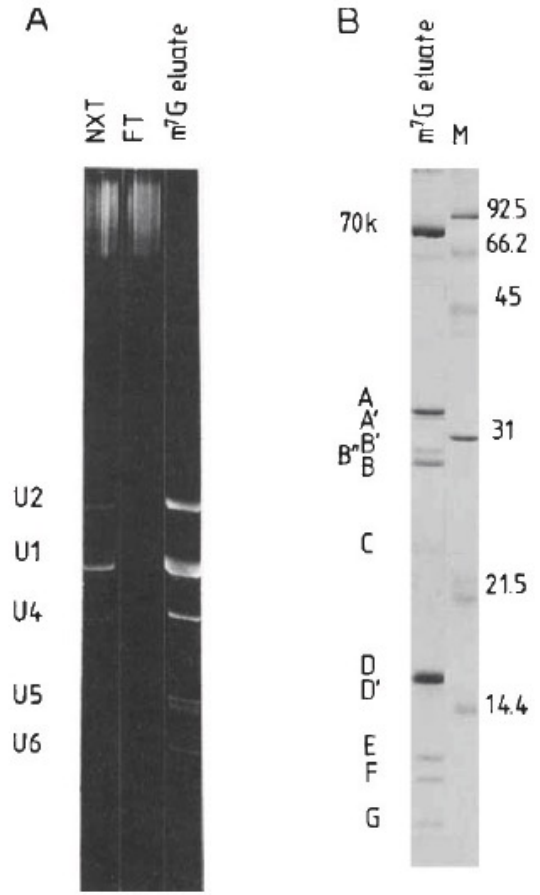
With specific antibodies or using tagged proteins



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

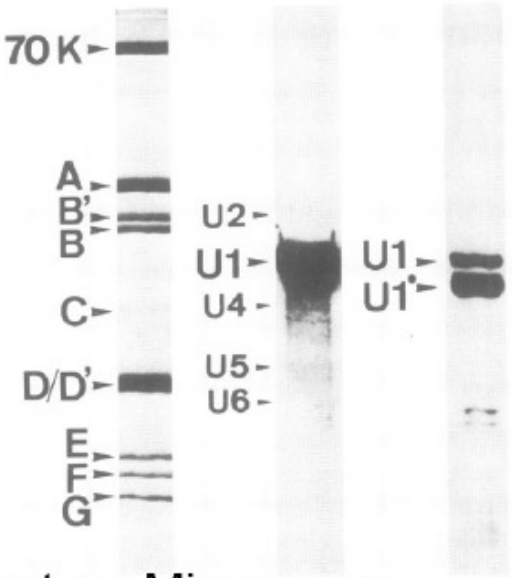


Bochnig et al, Eur. J Biochem. 1987
(Luhrmann's lab)

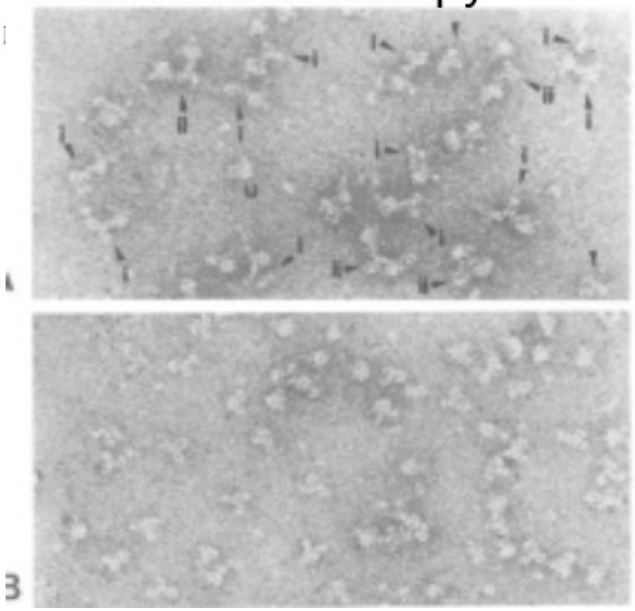
IP of U1 snRNP with α-70K

(U1 RNP specific protein)

Immunoaffinity +ion exchange



Electron Microscopy



IP of snRNPs with α-TMG cap

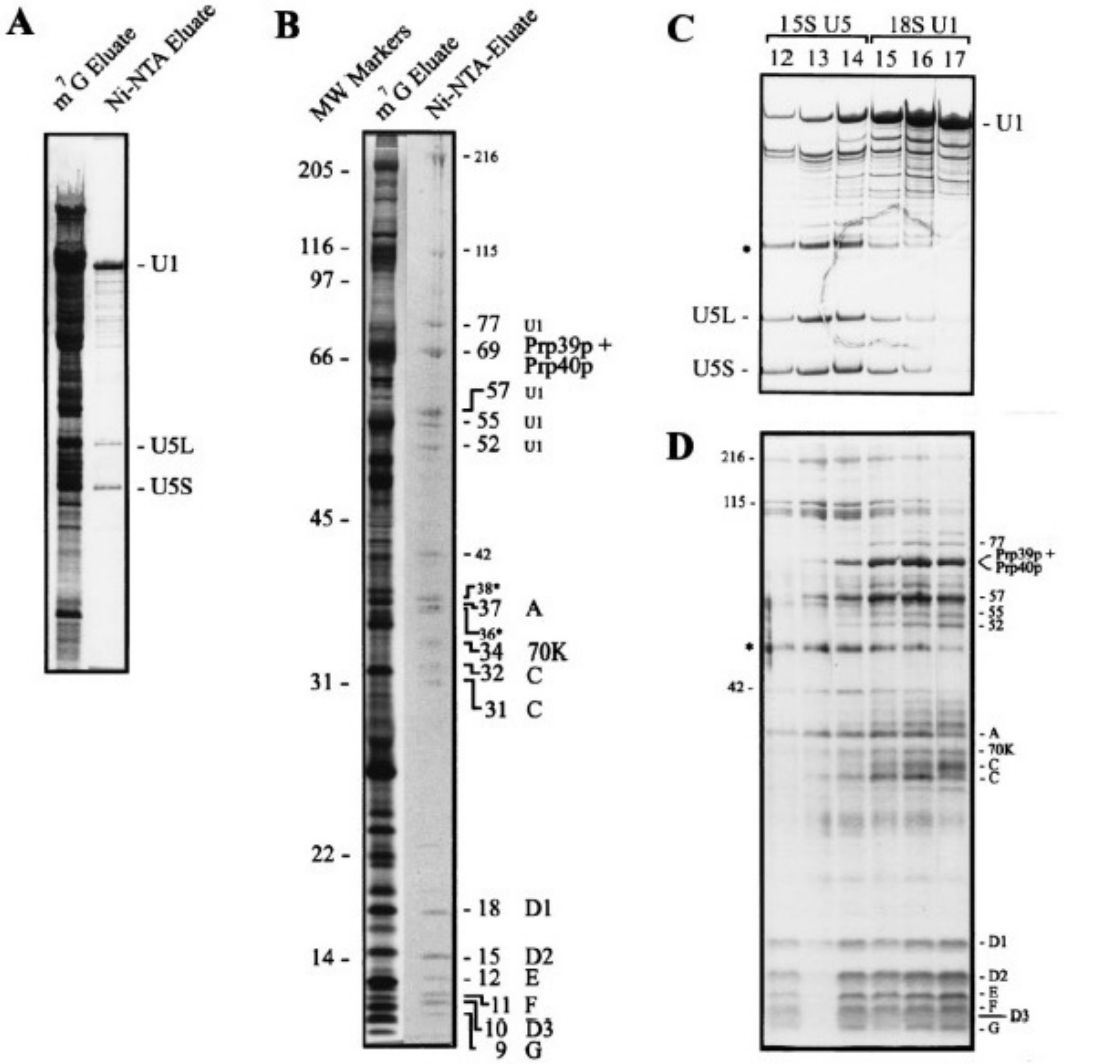
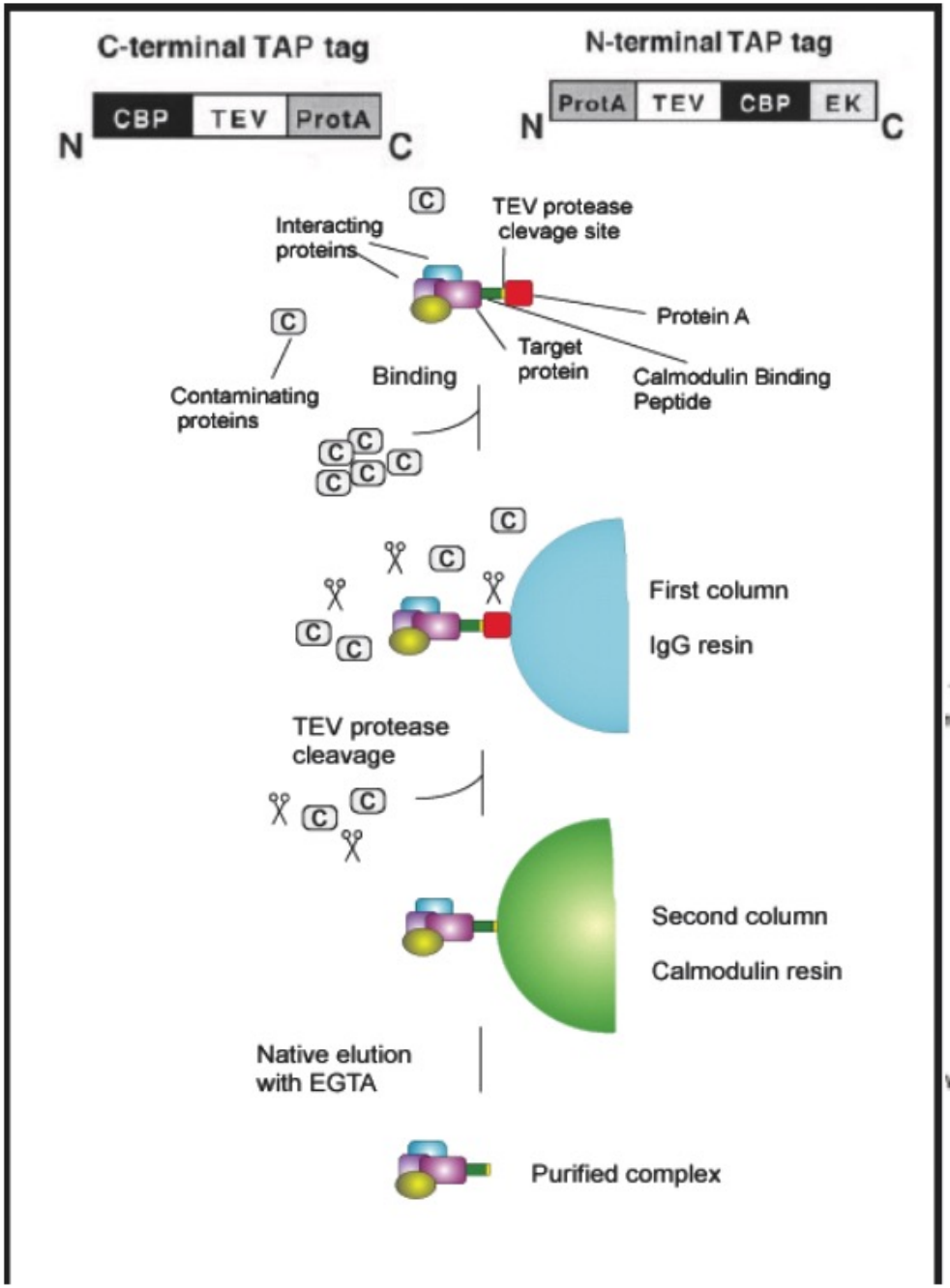
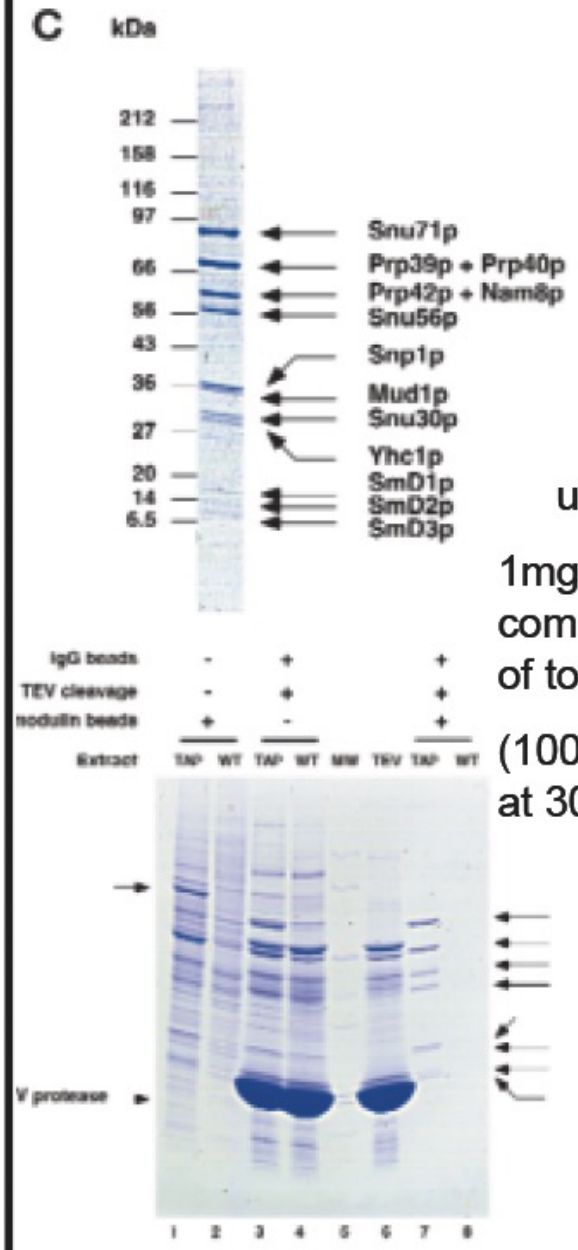


FIG. 1. Purification of U1 snRNPs from *S. cerevisiae*. (*A*) Silver staining of snRNAs eluted from anti-m_3G-cap (m^7G eluate) and Ni-NTA affinity

Tandem Affinity Purification (TAP)



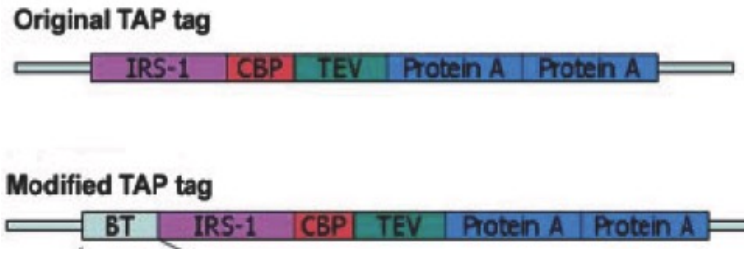


usual yield:

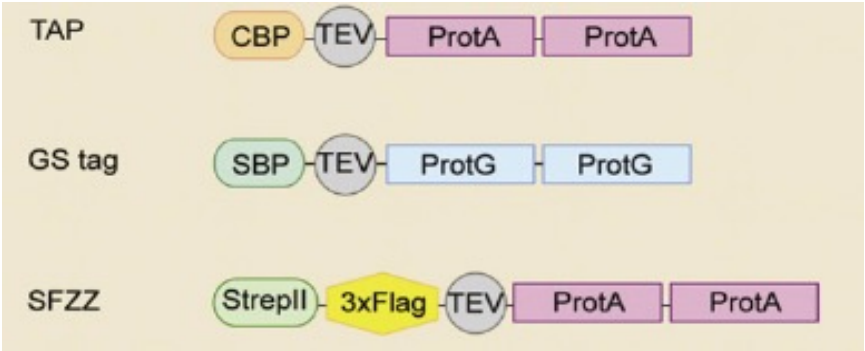
1mg of protein complex from 300mg of total protein

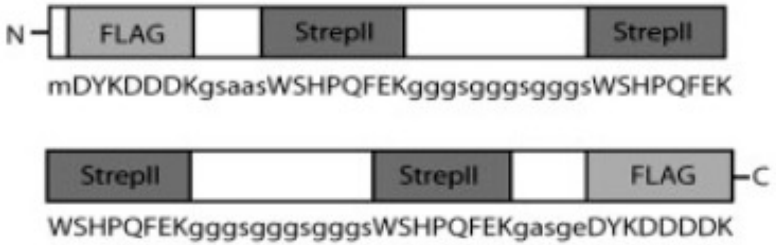
(100 000 X purification at 30% efficiency)

Modified TAP tags



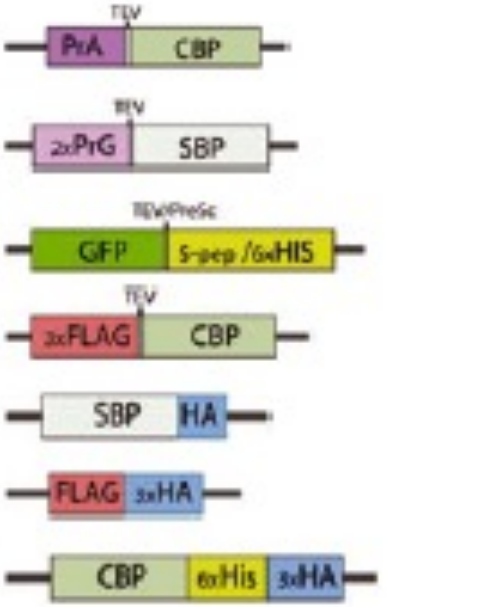
mammalian cells

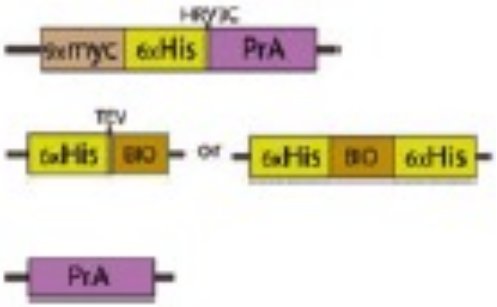




Drakas et al., Proteomics, 2005
Van Leene et al., TiPlSci, 2008;
Gloeckner et al, Proteomics, 2007
Oeffinger, Proteomics, 2012

Tandem

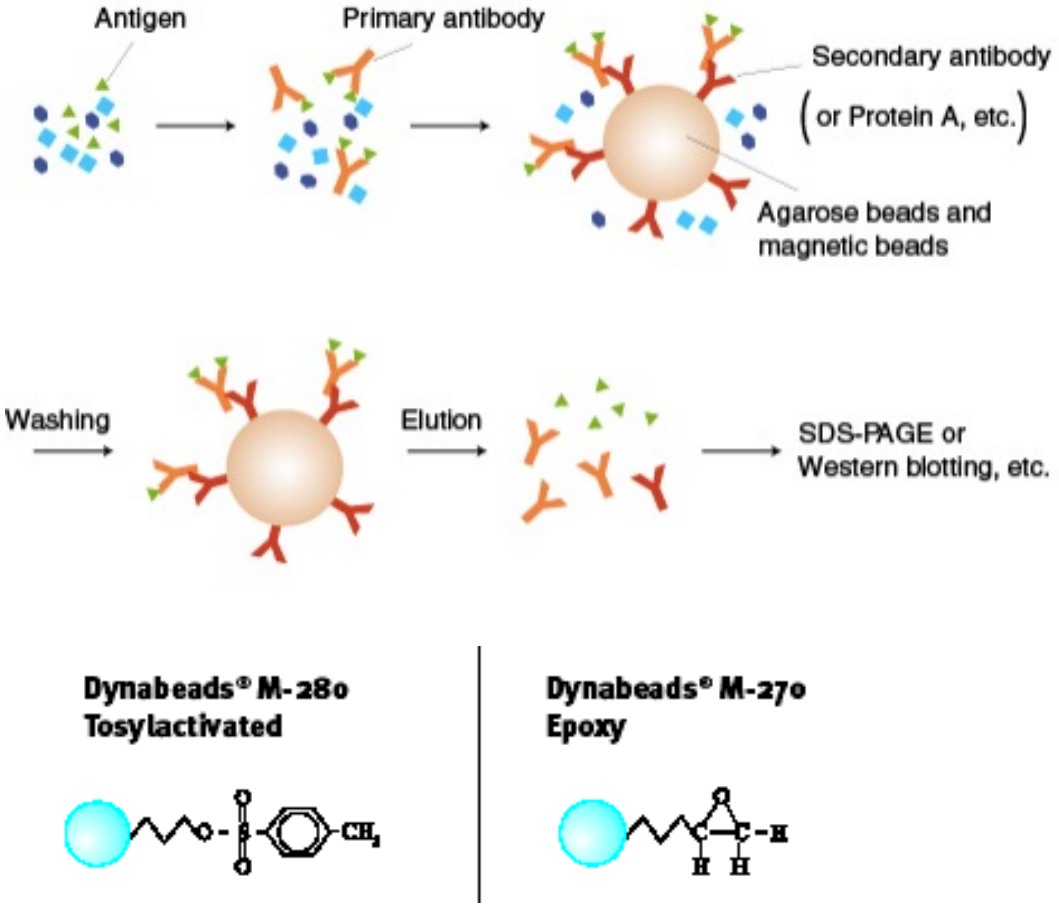


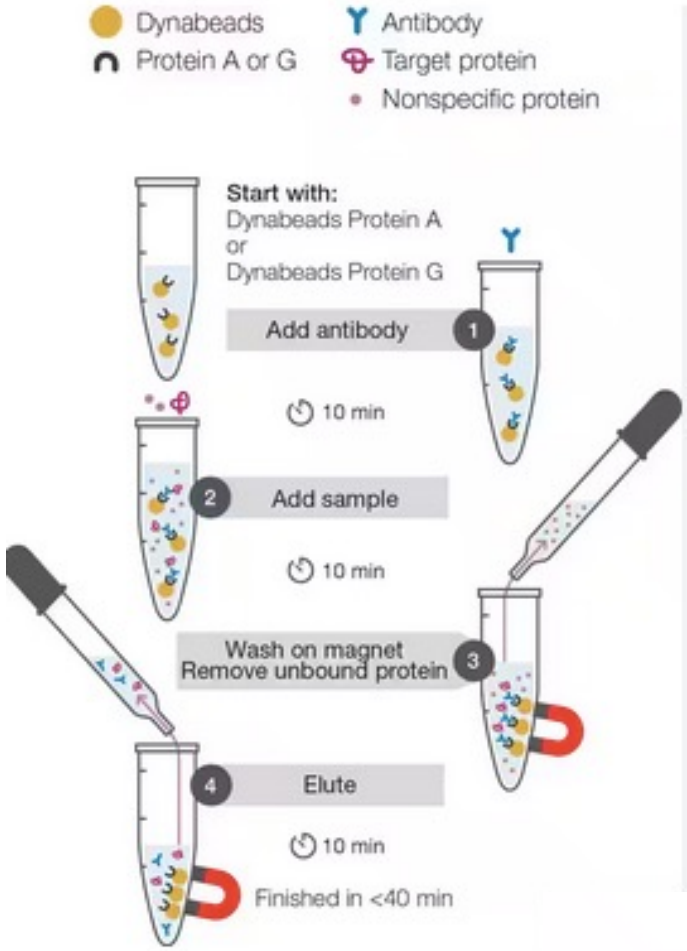


Single-step

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

CLIP

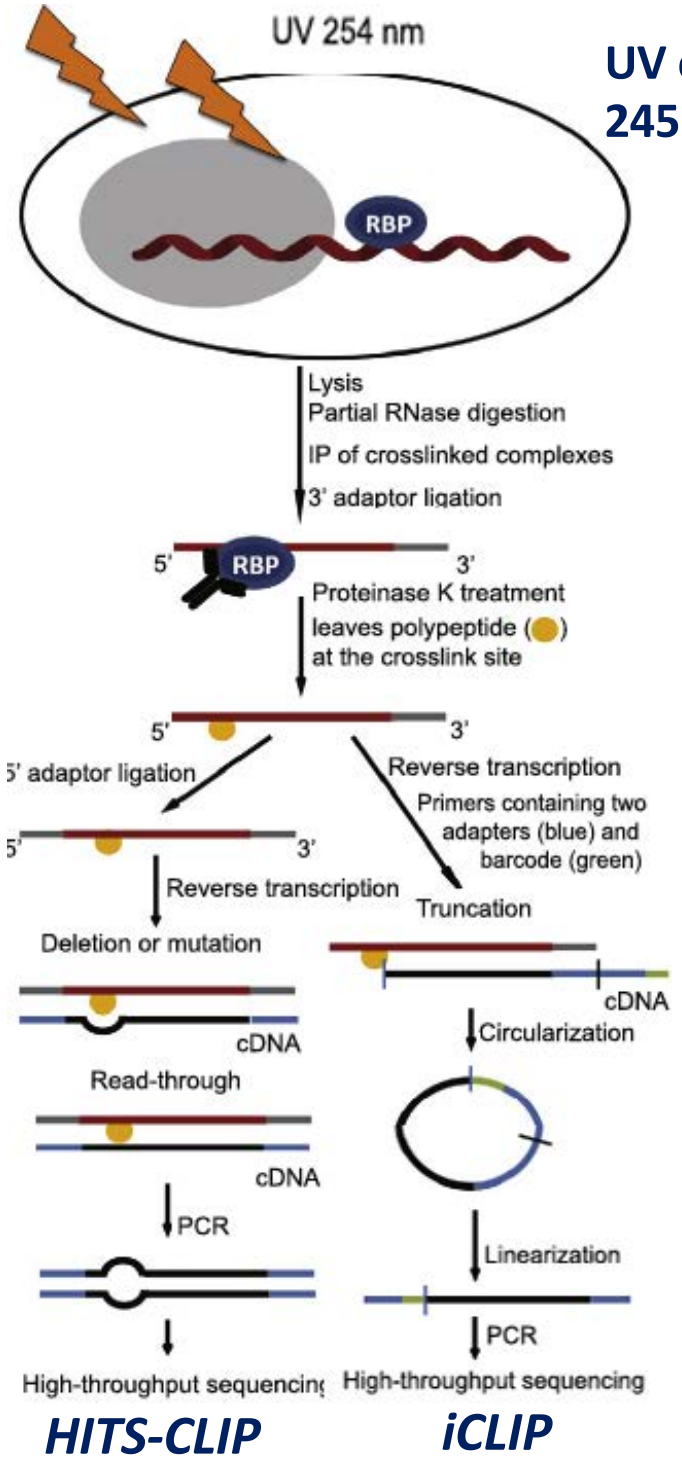
CrossLinking and ImmunoPrecipitation

HITS-CLIP

High-Throughput Seq CLIP

iCLIP

individual nucleoside resolution CLIP

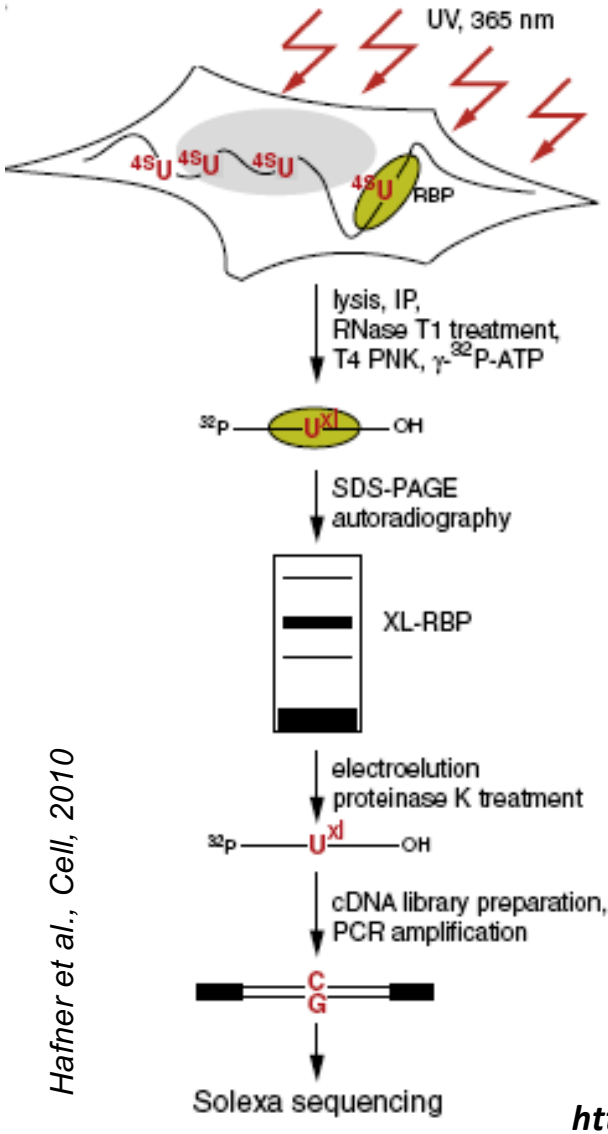


UV crosslinking
245 nm

Li et al., Genome Proteome Bioinformatics, 2014

PAR-CLIP

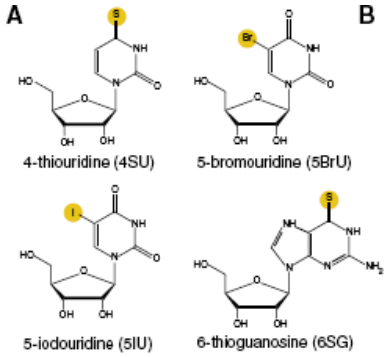
PhotoActivatable ribonucleoside–enhanced CLIP

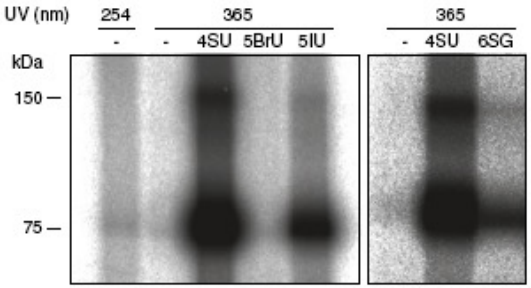


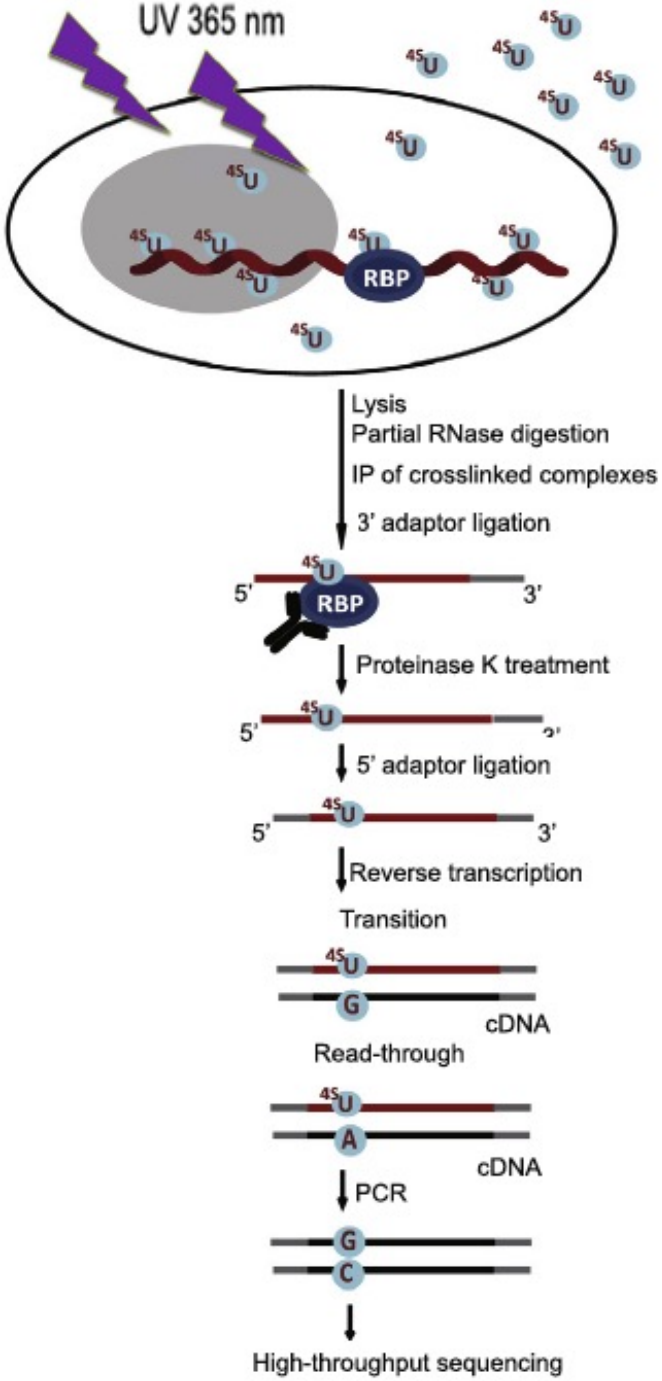
Hafner et al., Cell, 2010

RNA labeling with U derivatives

UV crosslinking 365 nm

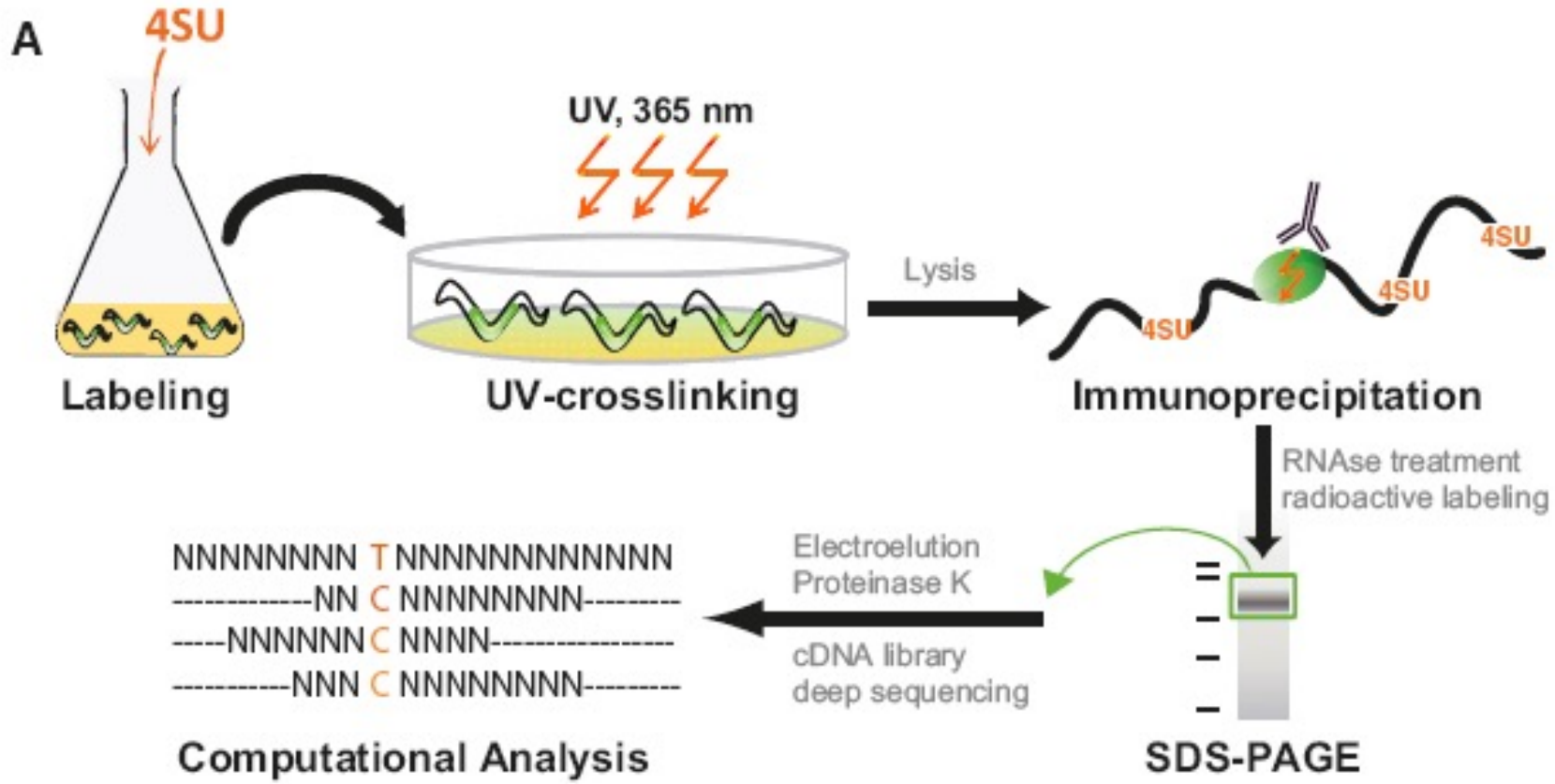






http://www.jove.com/index/details.stp?ID=2034

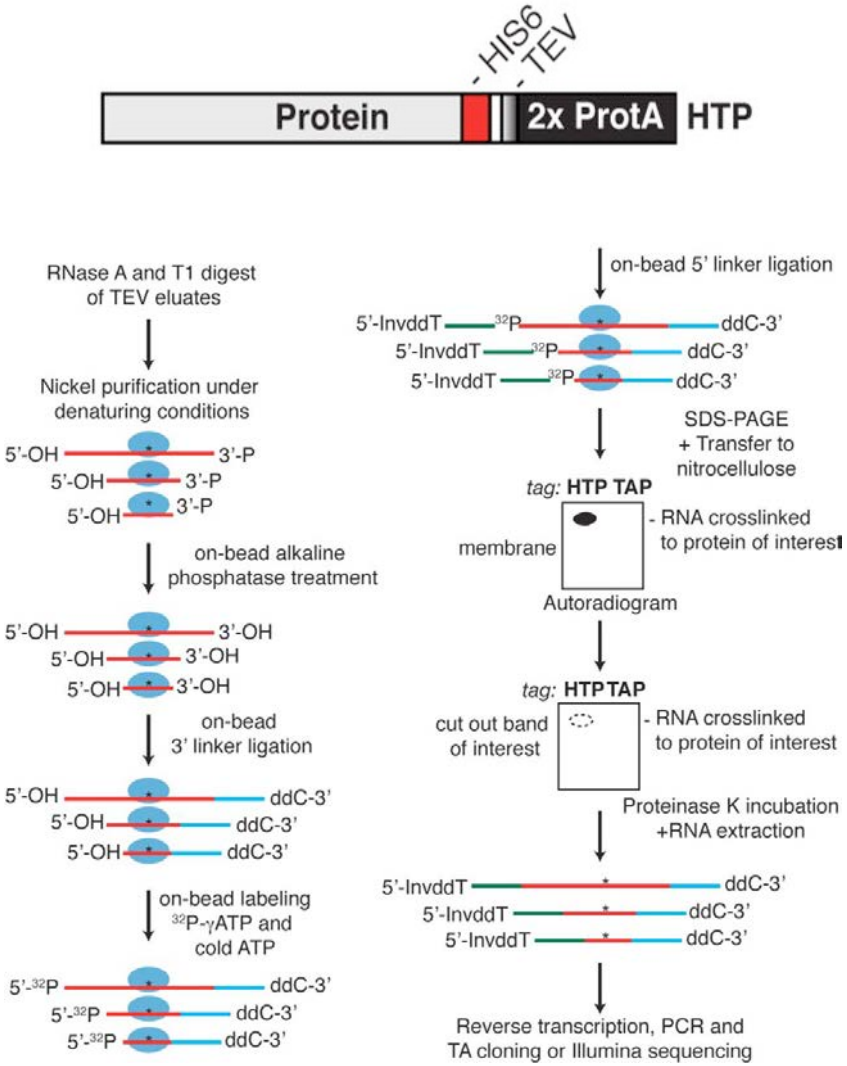
in vivo PAR-CLIP

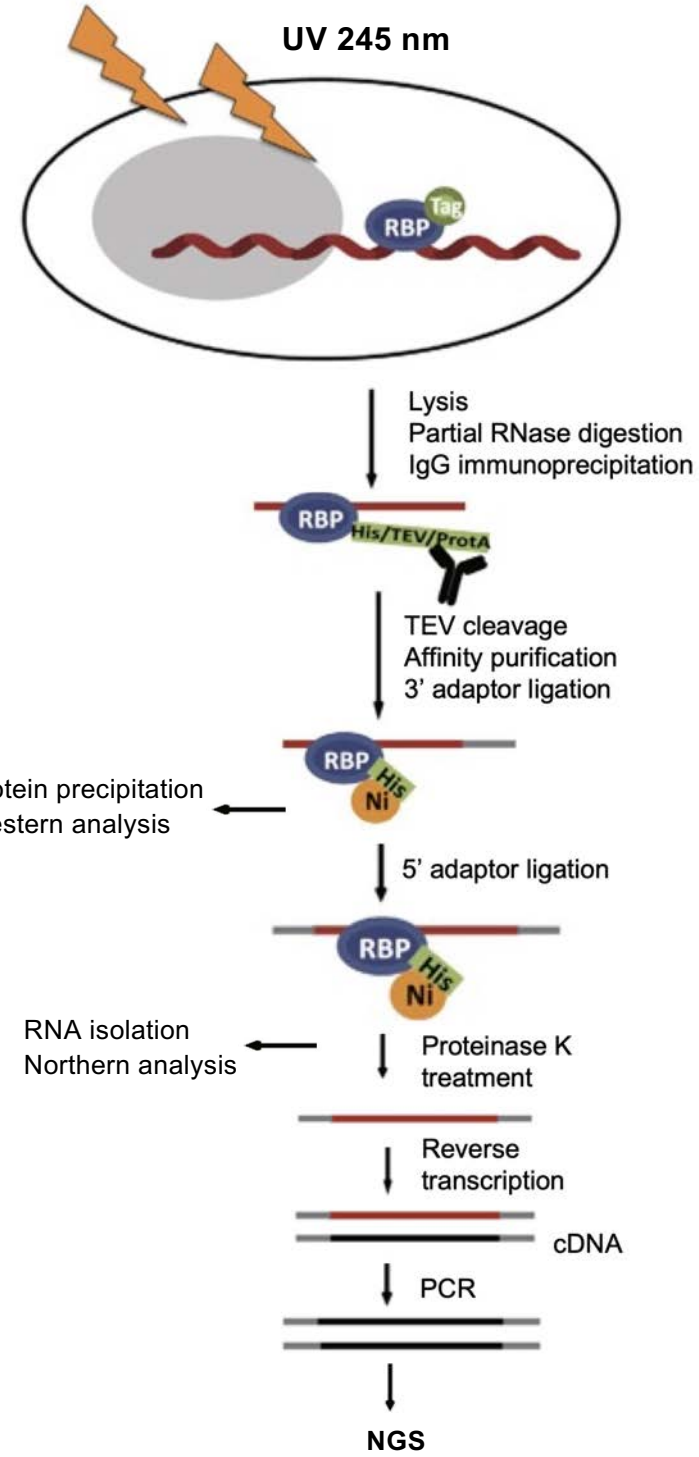


Jungkamp et al., Mol Cell, 2011

CRAC

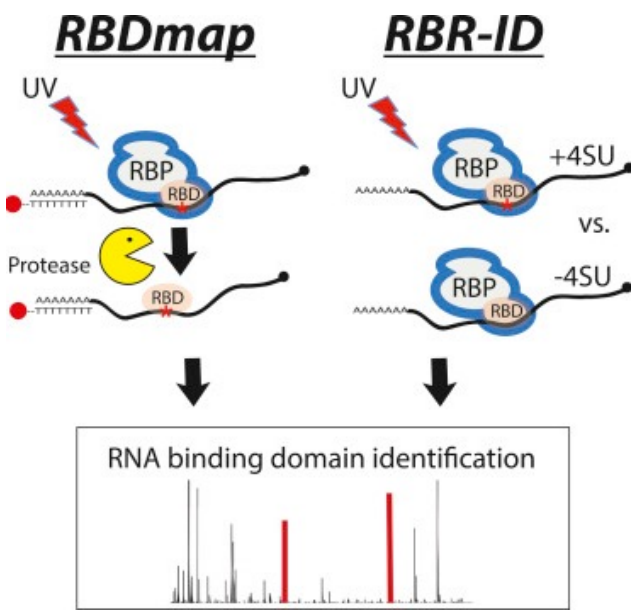
CRosslinking and Analysis of cDNA

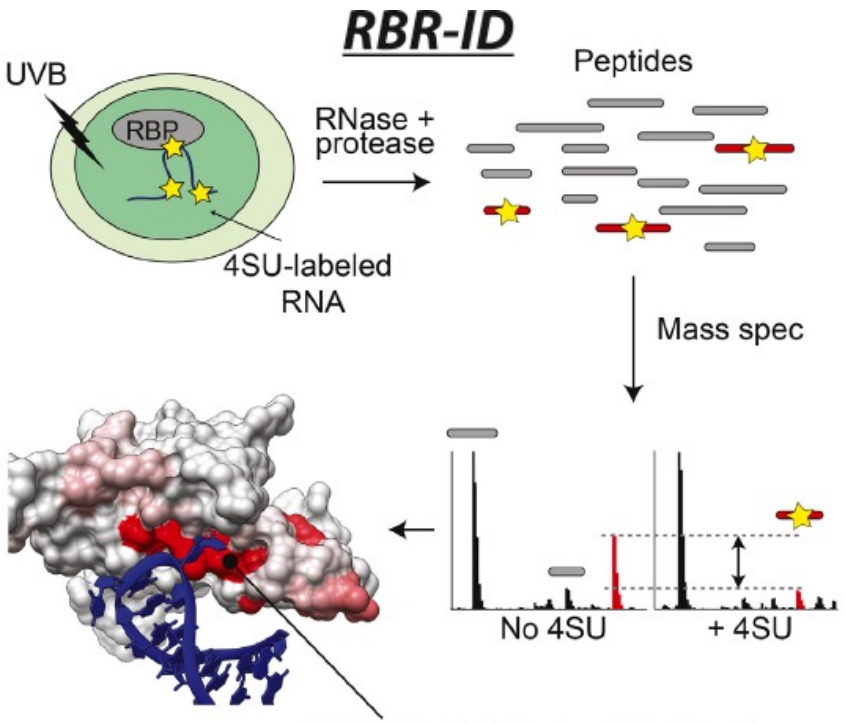


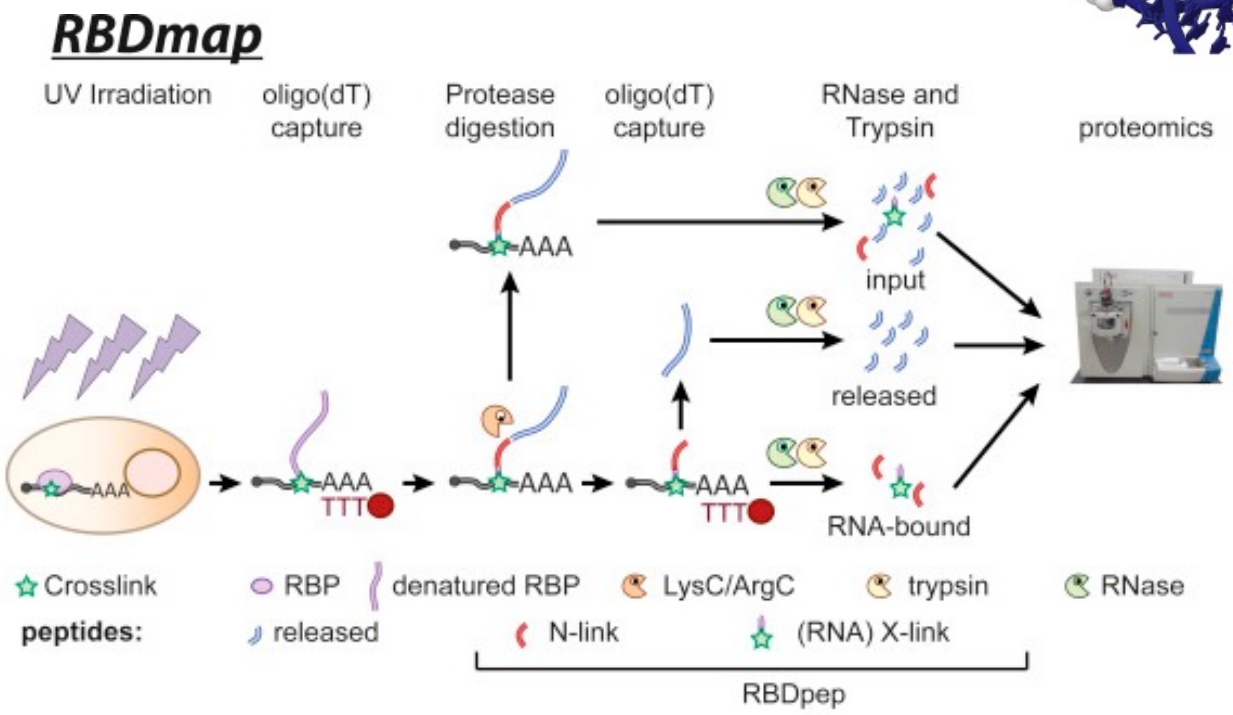


Granneman et al., PNAS, 2009
Li et al., Genome Proteome Bioinformatics, 2014

mRNA binding proteome

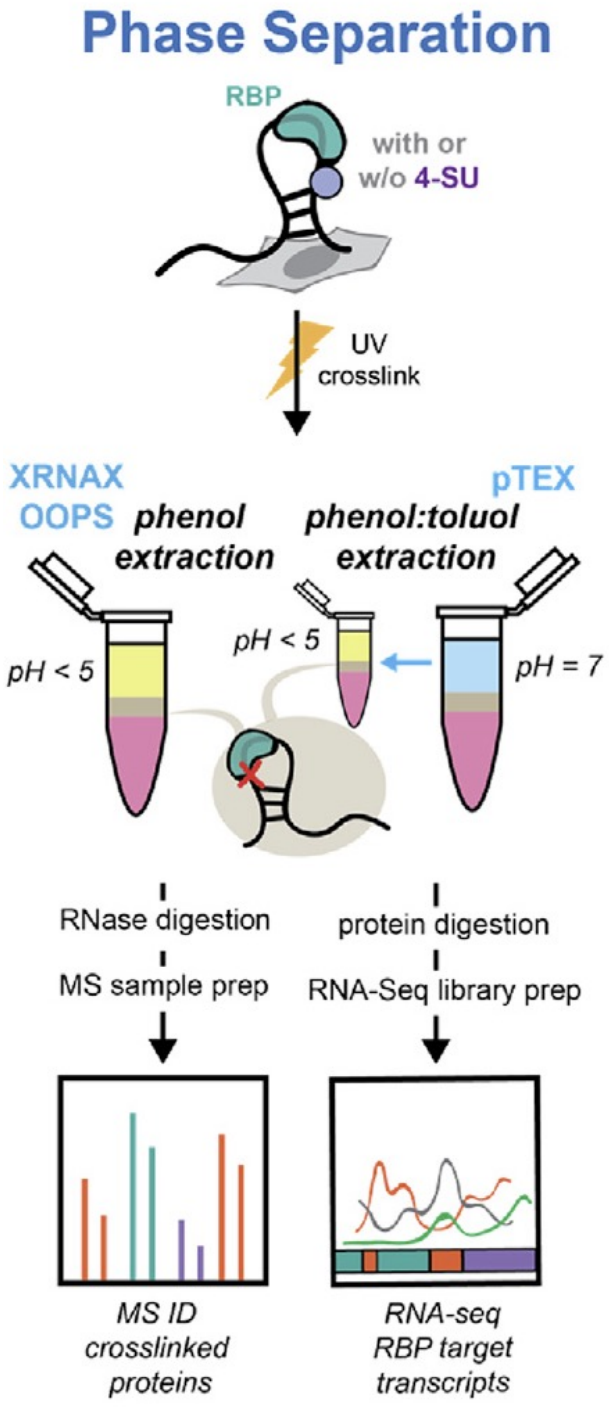






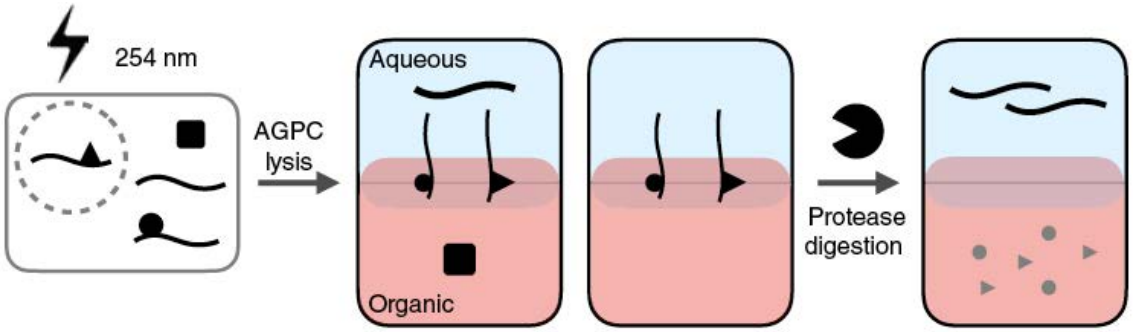
Identification of poly(A) RNA binding proteins

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

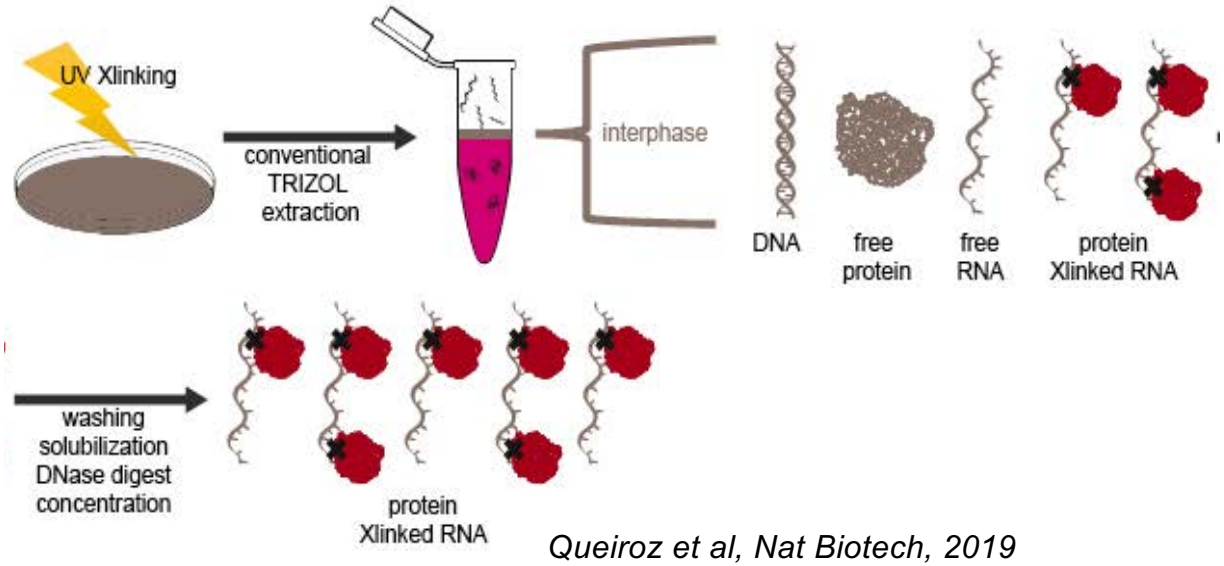


OOPS, XRNAX, pTEX
RNP interactome, RPBome

OOPS - orthogonal organic phase separation

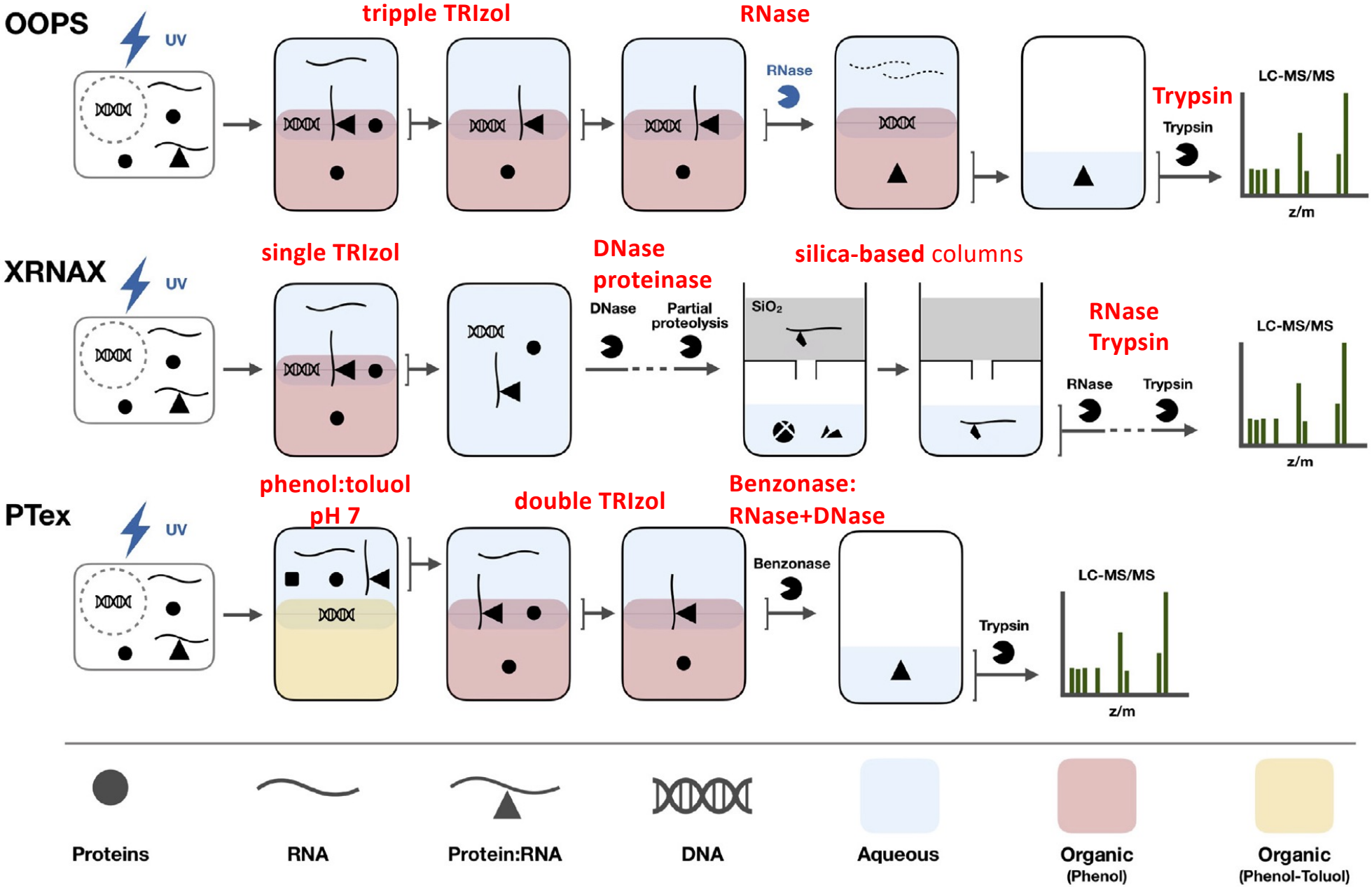


XRNAX - Cross-Linked RNA eXtraction



Queiroz et al, Nat Biotech, 2019
Shchepachev et al, Mol Sys Biol, 2019
Nechay and Kleiner, Curr Op Chem Biol, 2020

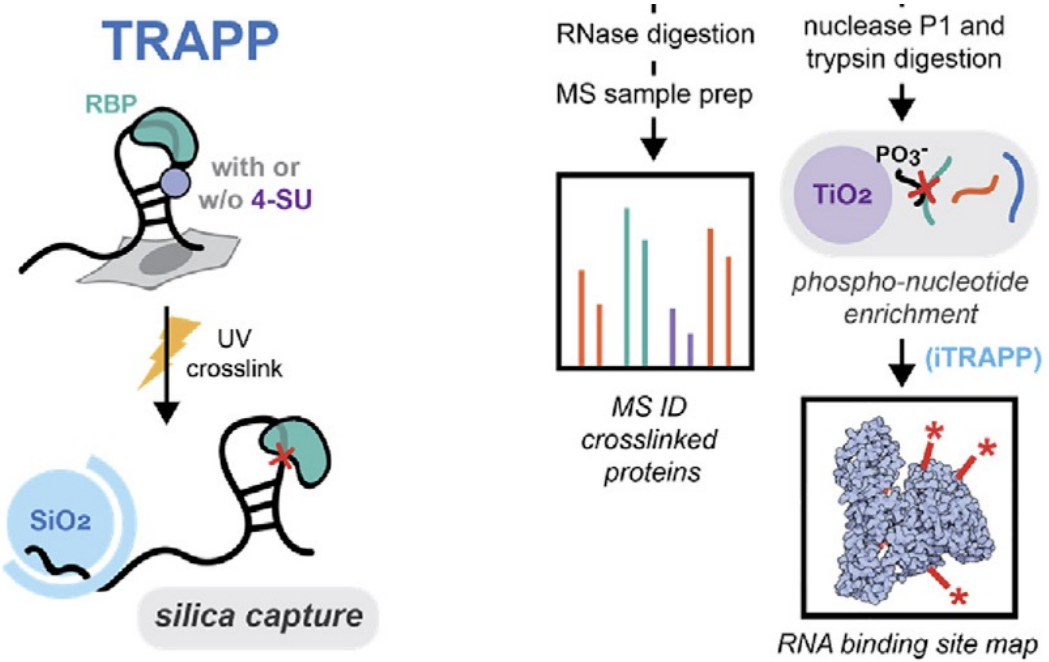
OOPS, XRNAX, PTex – organic phase separation



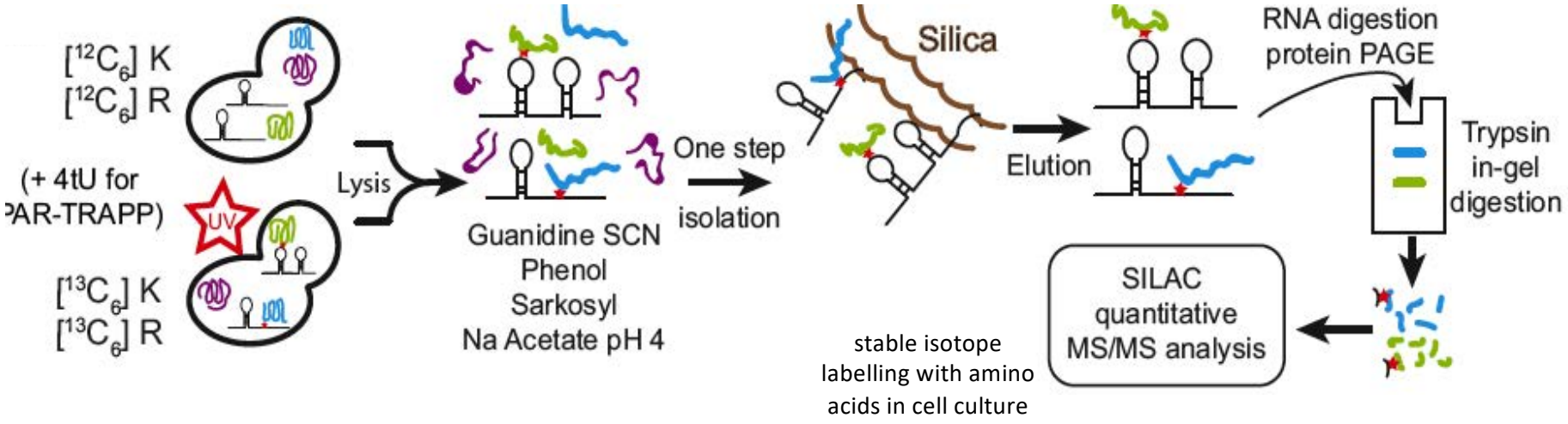
Smith et al. Curr Op Chem Biol, 2020

TRAPP RNP interactome, RPBome

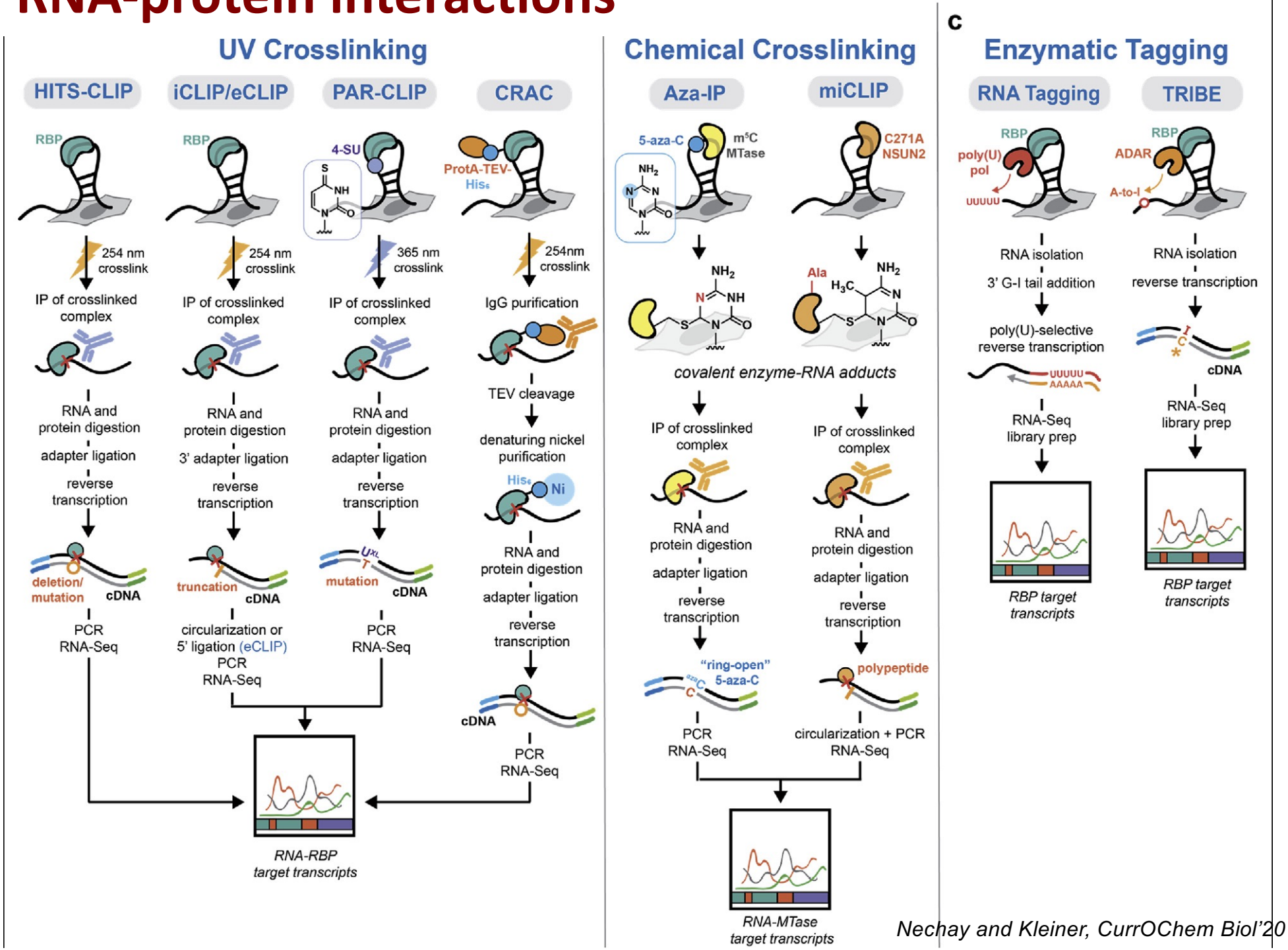
TRAPP/PAR-TRAPP - RNA-associated protein purification



Queiroz et al, Nat Biotech, 2019
Shchepachev et al, Mol Sys Biol, 2019
Nechay and Kleiner, Curr Op Chem Biol, 2020



RNA-protein interactions

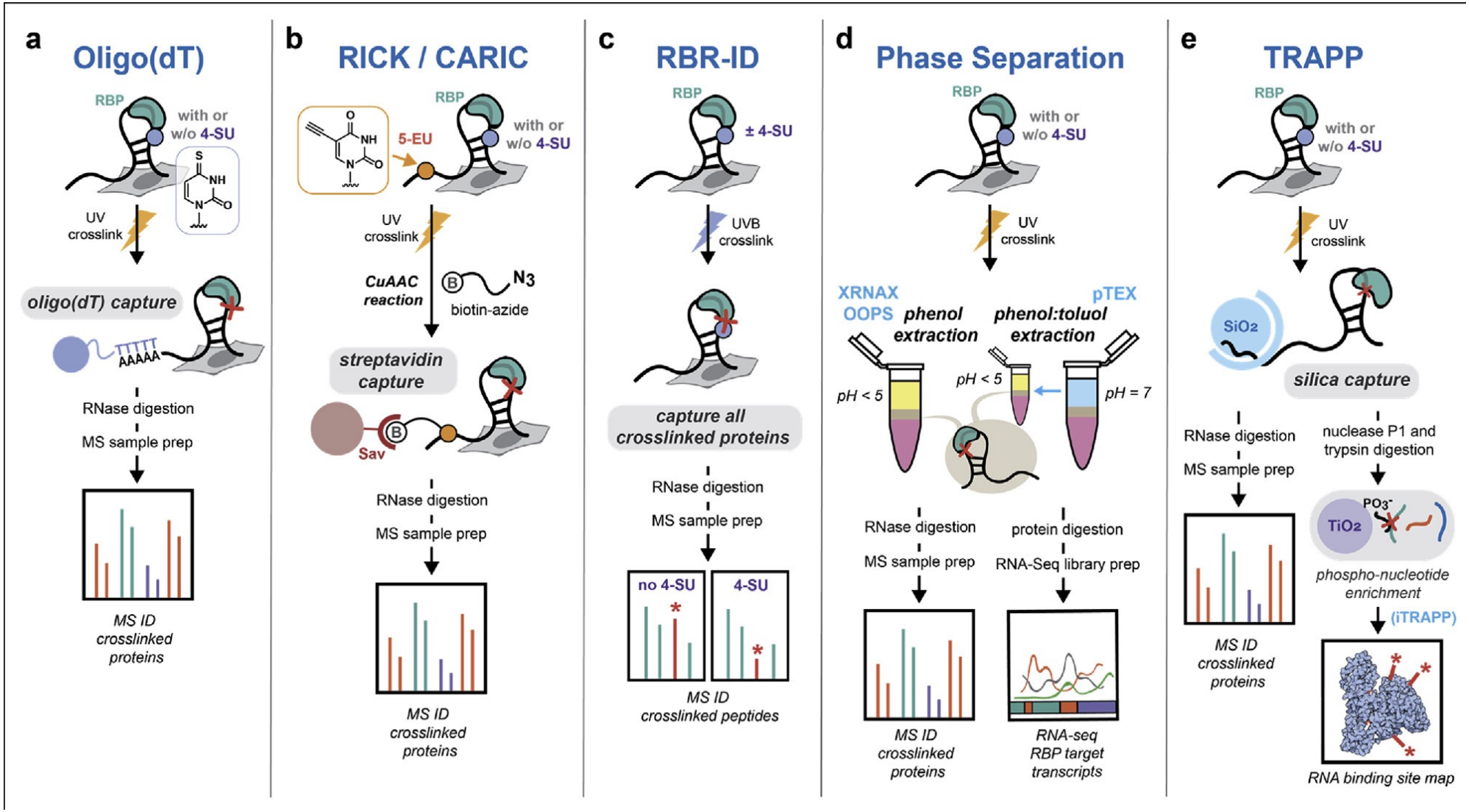


Nechay and Kleiner, CurrOChem Biol'20

RNA-protein interactions

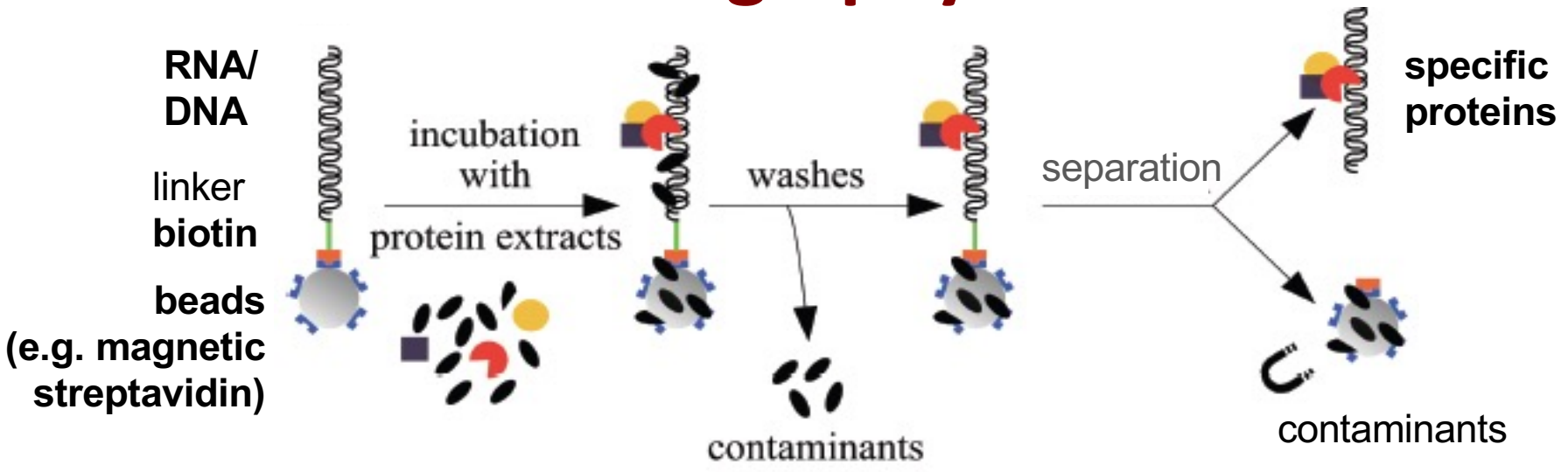
Nascent RNA can be labeled with 4-thioU (4-SU) or 6-thioG (6-S

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

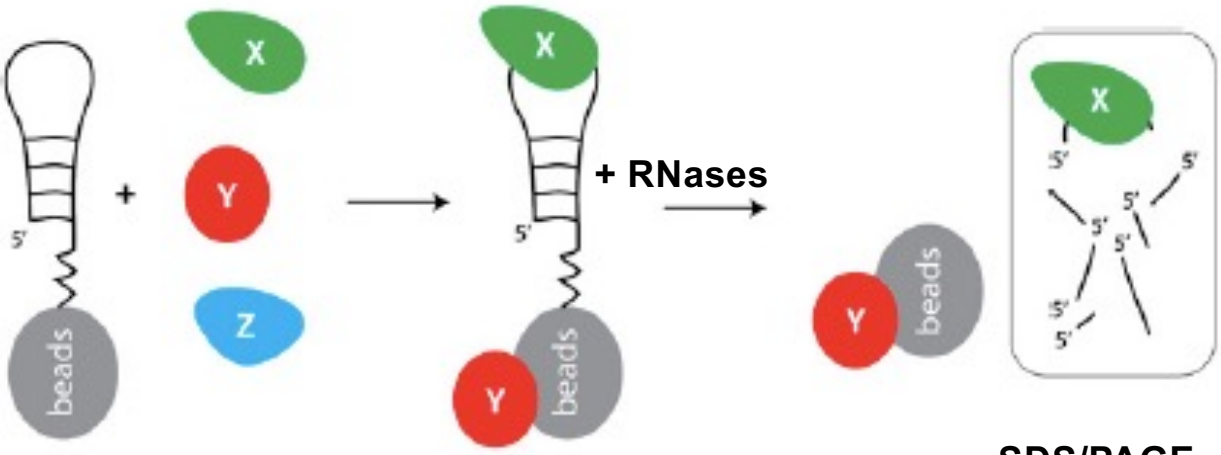


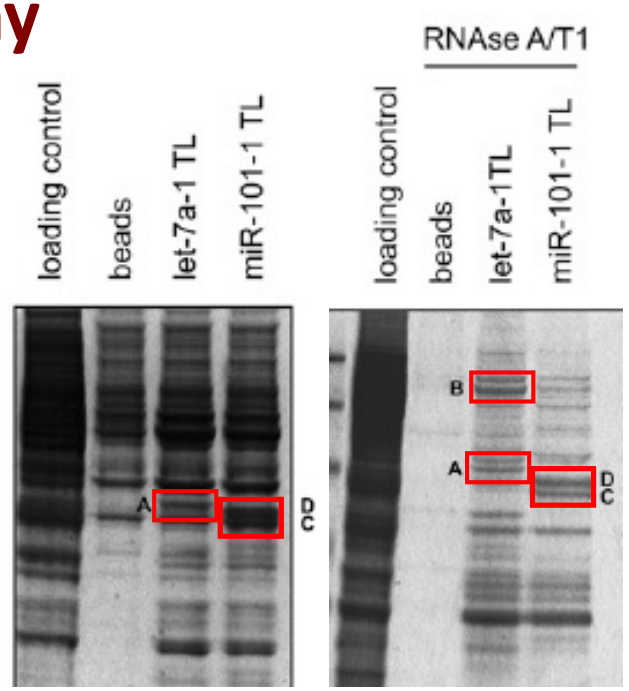
Nechay and Kleiner, CurrOChem Biol'20

RNA chromatography *in vitro*



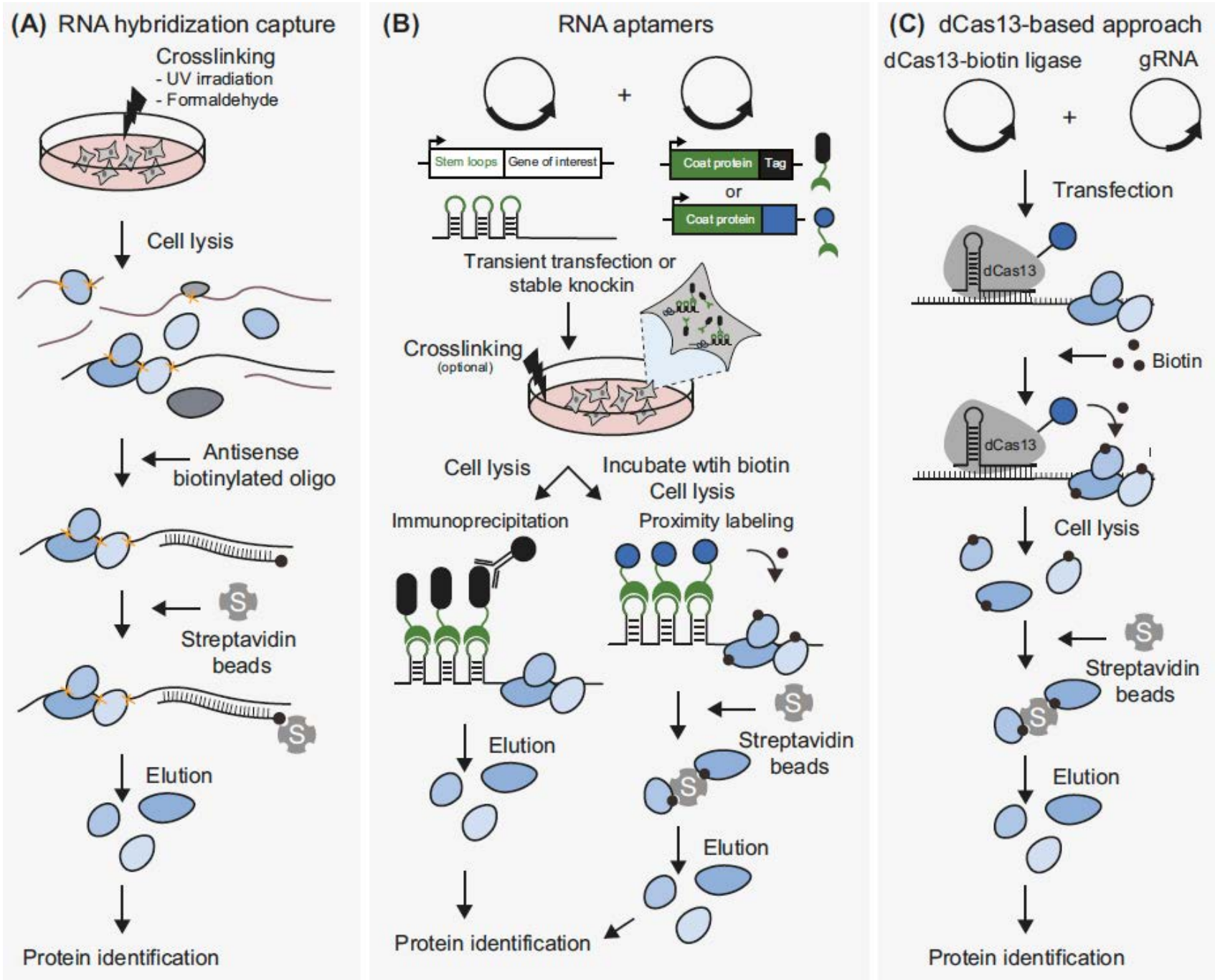
RNase-assisted RNA chromatography





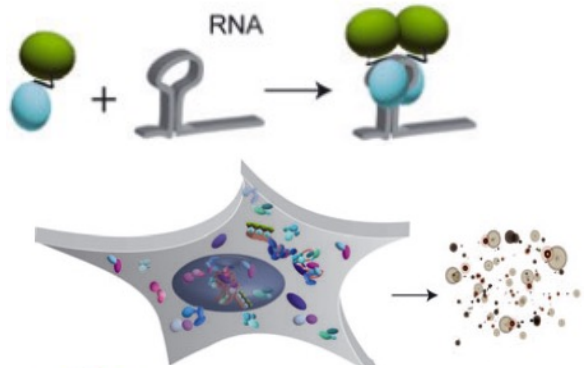
Hegarat et al., NAR, 2010; Michlewski and Caceres, RNA, 2010

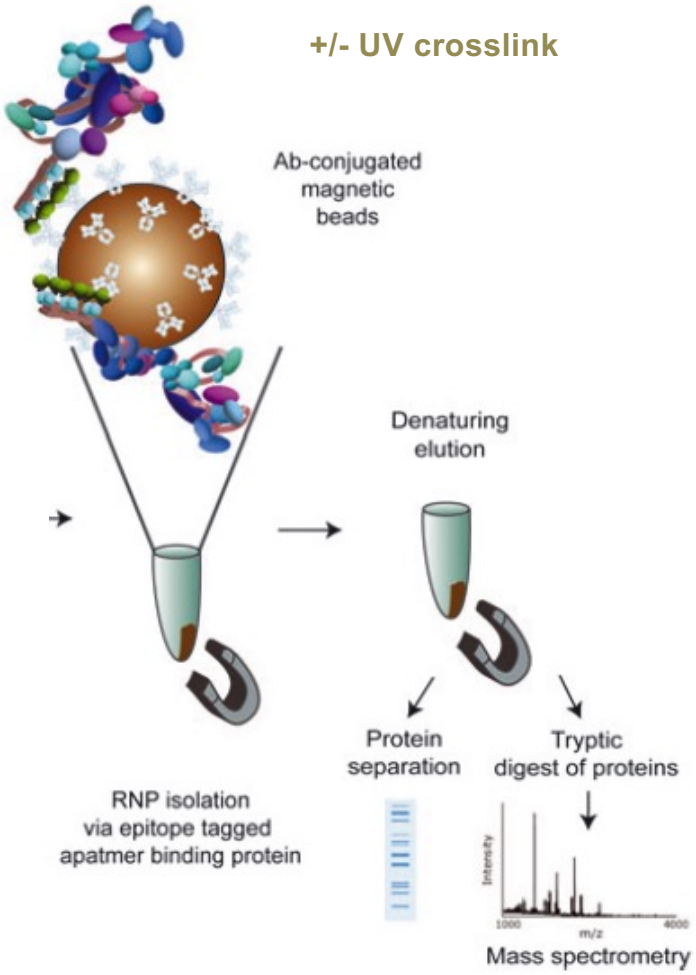
RNA chromatography *in vivo*



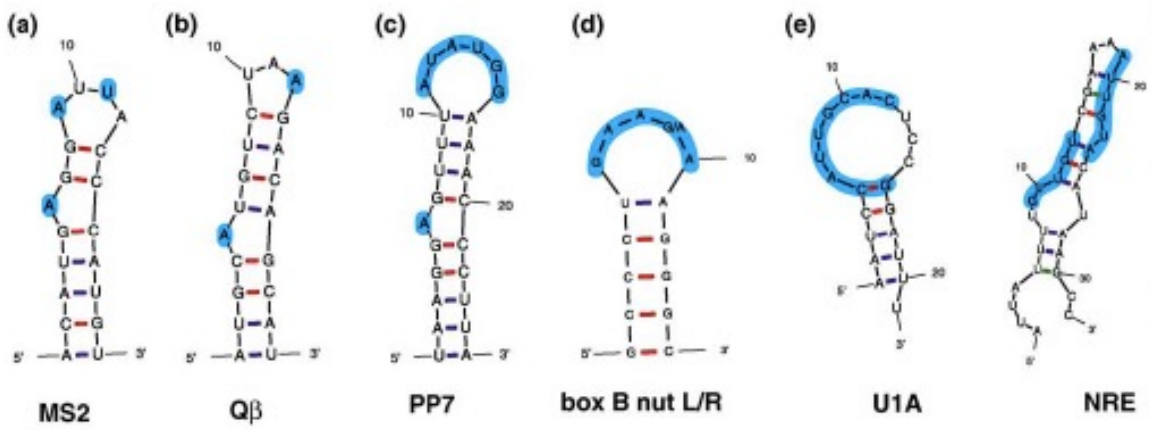
Gräwe et al, Trends in Biotech 2021

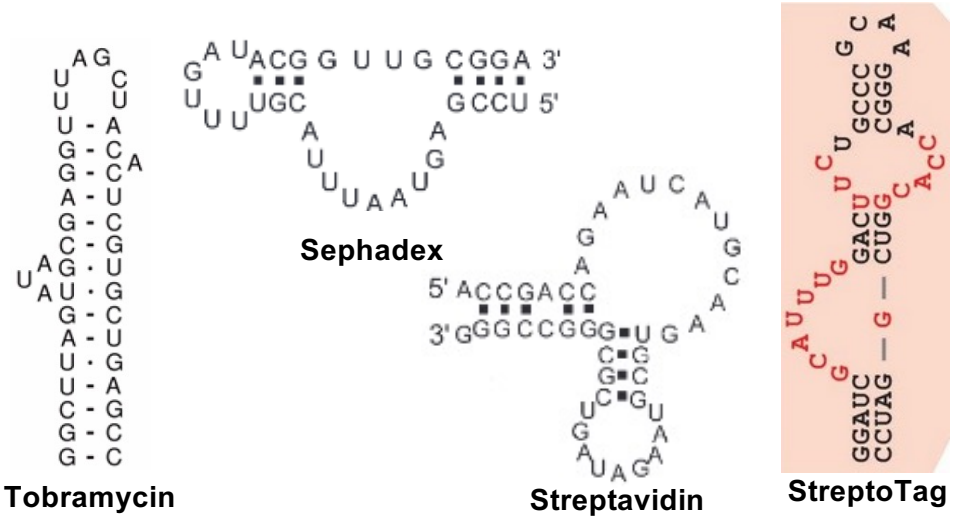
RNA chromatography *in vivo*





Oeffinger, *Proteomics*, 2012





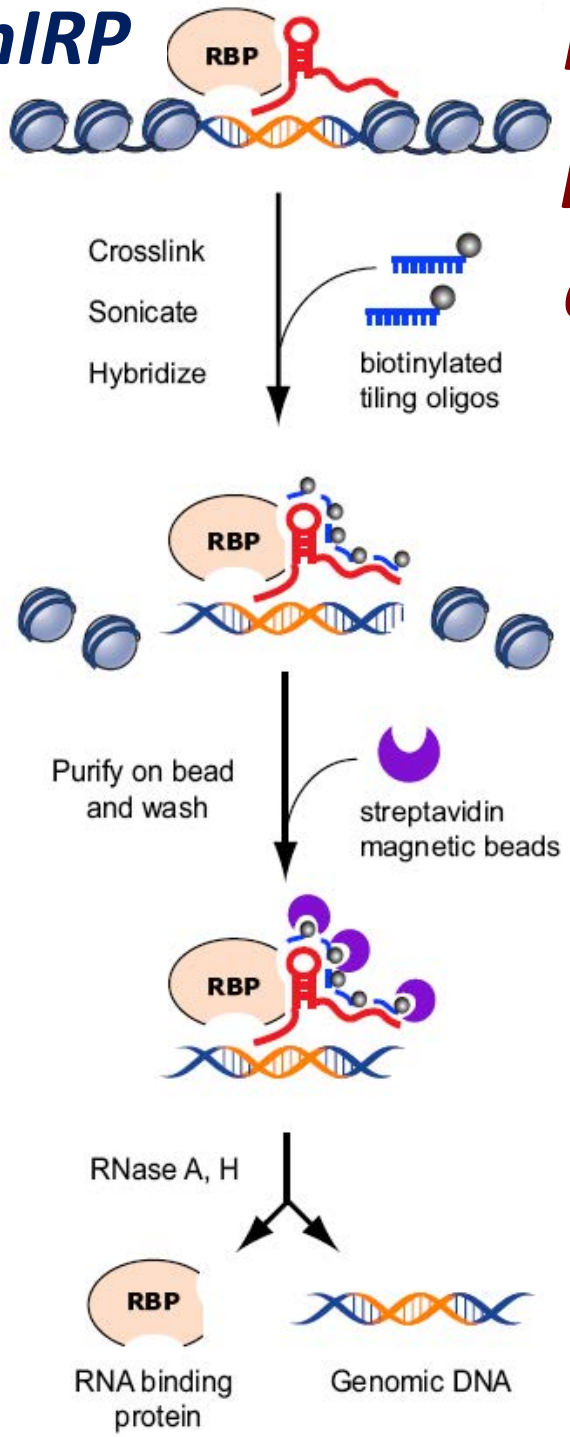
RNP purification from cells expressing RNA with affinity tags that bind to specific proteins or resins.

Hartmuth et al, Meth Mol Biol, 2004; Walker at al, Meth Mol Biol 2008; Windbichler, Schroeder, Nat Protocol, 2006

lncRNA-proteins-chromatin

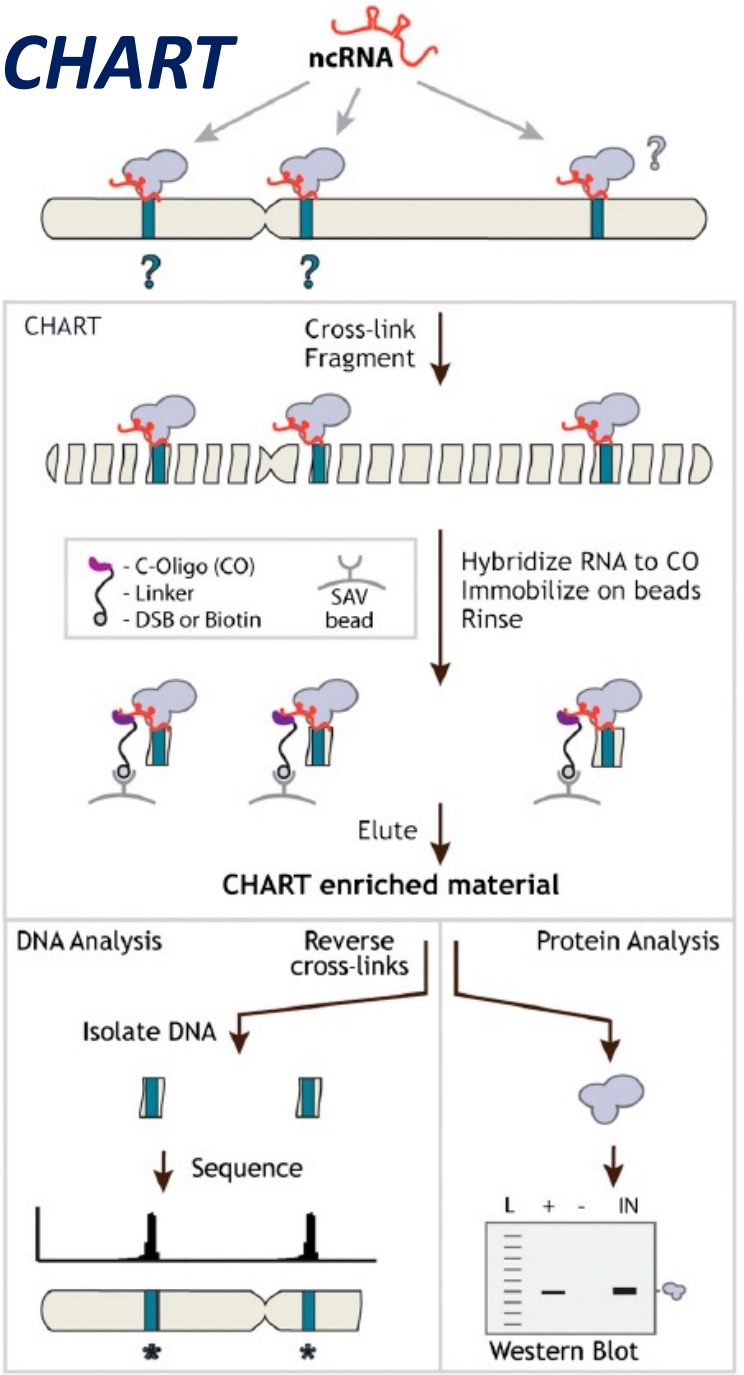
ChIRP

Chromatin Isolation by RNA Purification



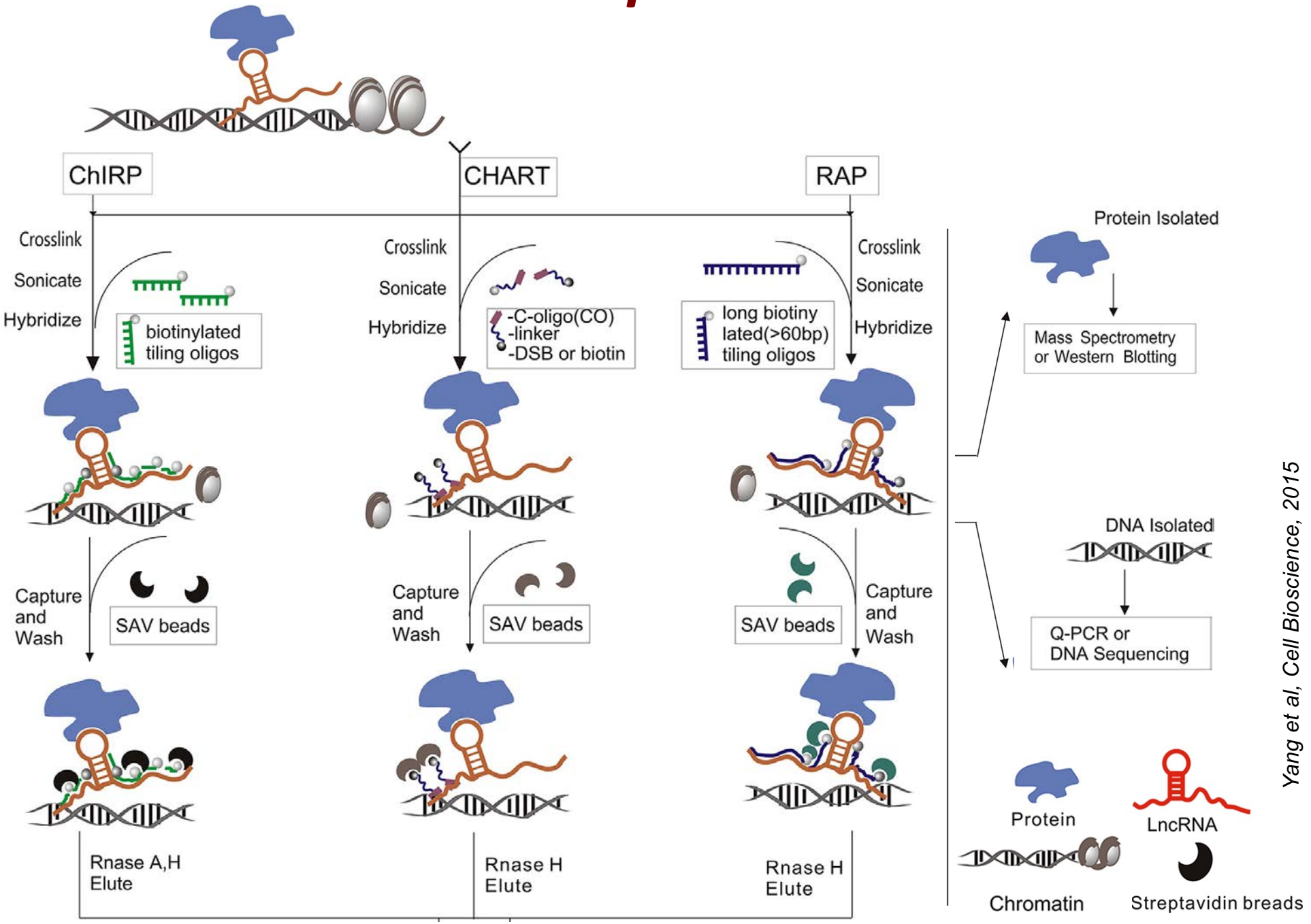
CHART

Capture Hybridization Analysis of RNA Targets

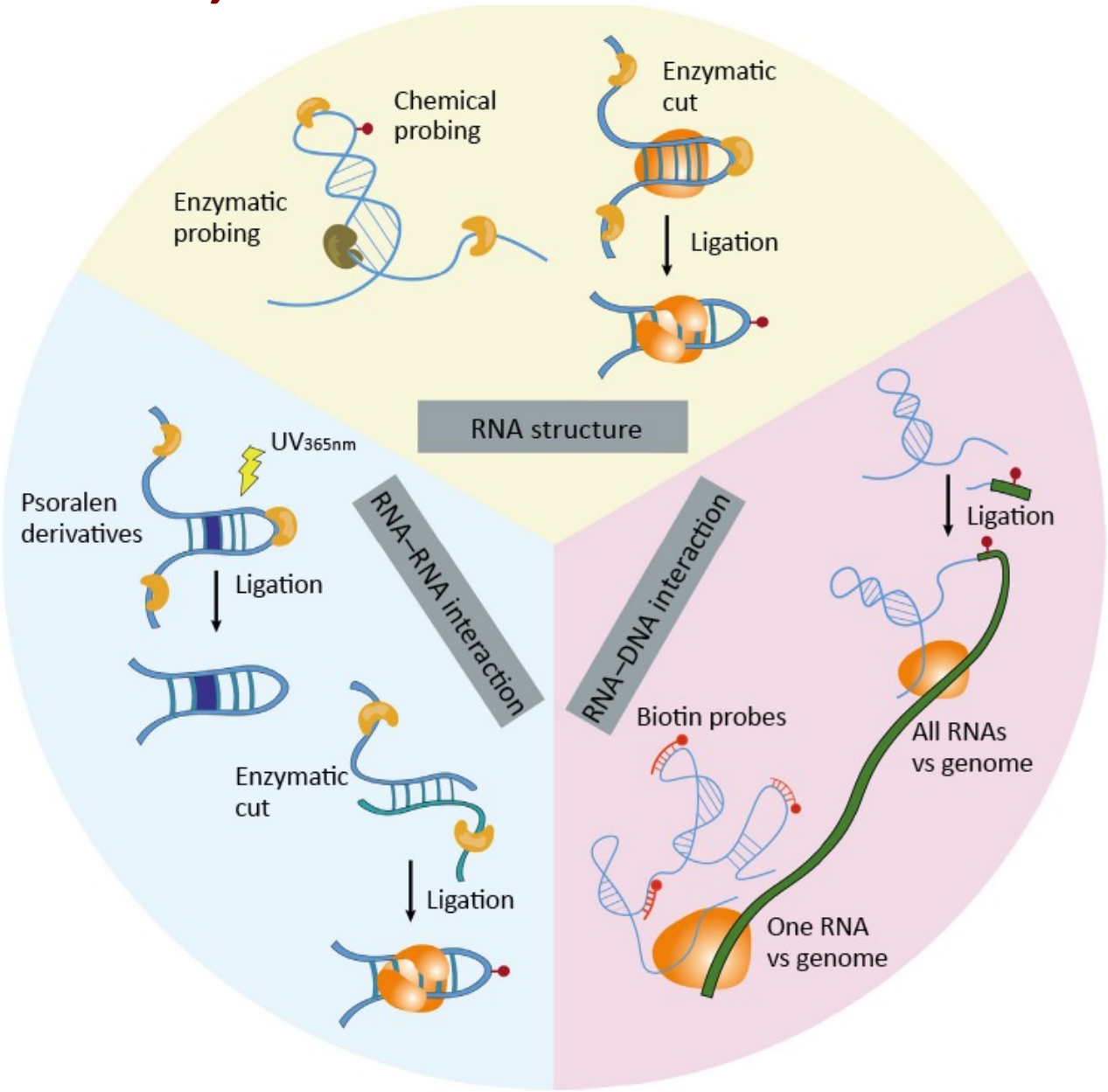


Chu et al., Mol. Cell, 2011; Simon et al., PNAS '11

Interactions lncRNA-proteins-chromatin



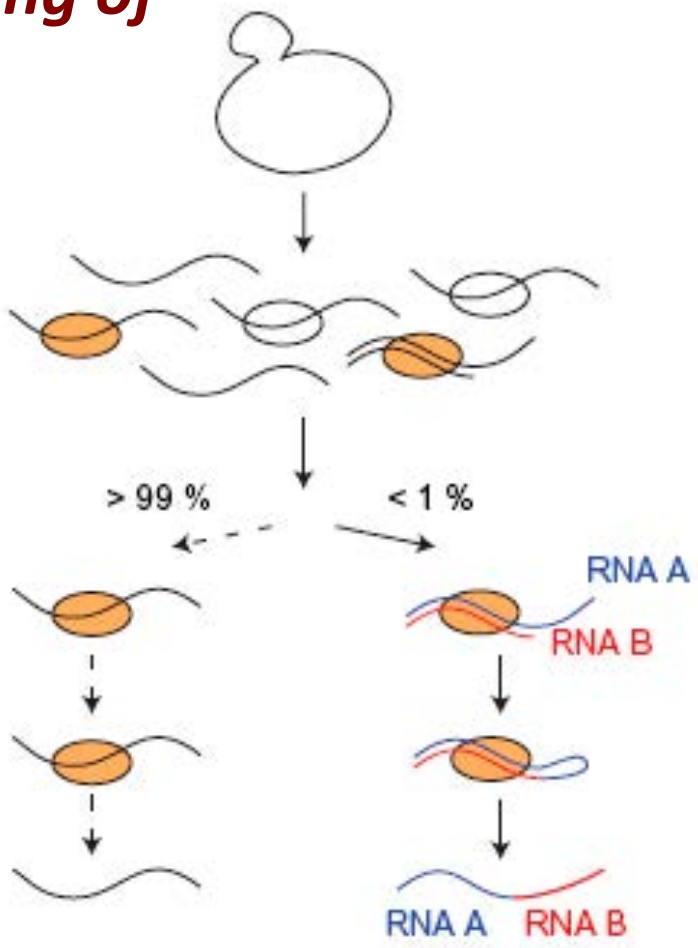
RNA-seq-based methods for mapping RNA structures, RNA–RNA and RNA–DNA interactions

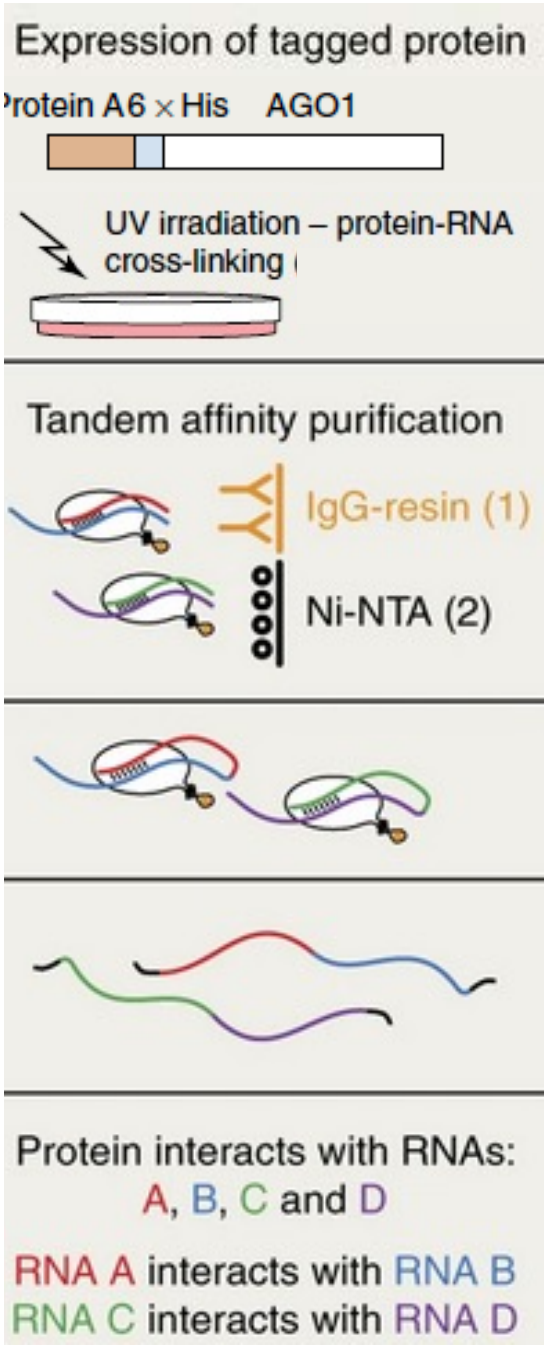


Nguyen et al, *TiG, 2018*

Intra- and intermolecular RNA-RNA interactions CLASH

Crosslinking Ligation and Sequencing of Hybrids





Kudla et al., PNAS, 2011; Helwak and Tollervey, Nat Protocol 2014

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

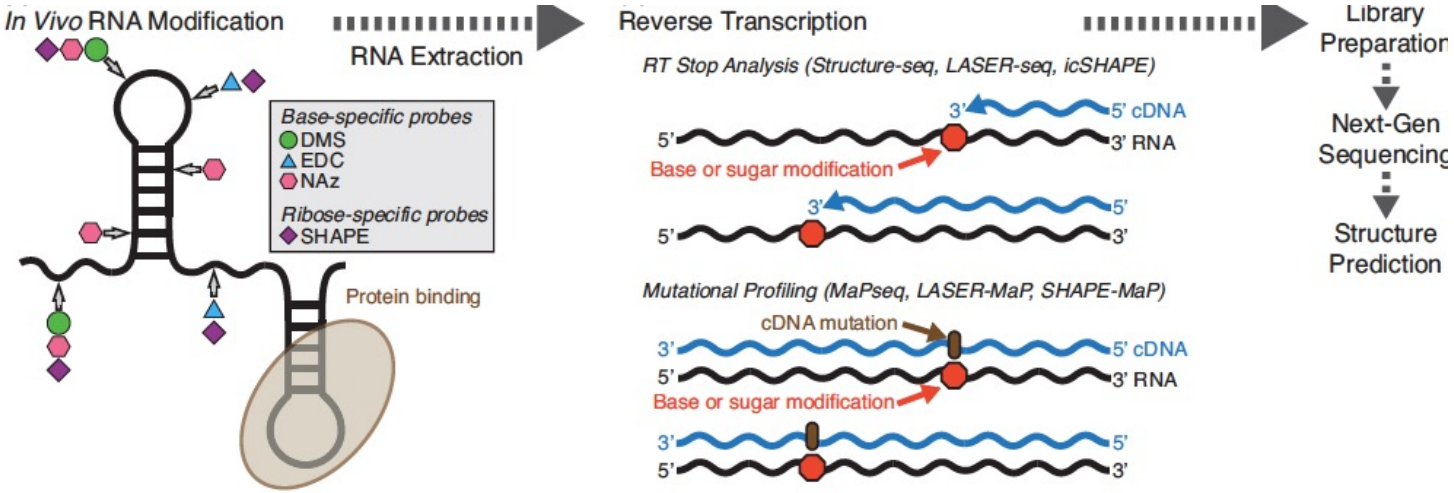
SHAPE chemicals: DMS, dimethyl sulfate; 1M7, 1-methyl-7-nitroisatoic anhydride
SHAPE enzymes: P1 nuclease, RNases V1 and S1
PARIS/SPLASH chemicals: psoralen; AMT, 4'-aminomethyltrioxsalen

Table 1. Transcriptome-wide RNA Structure Probing Methods

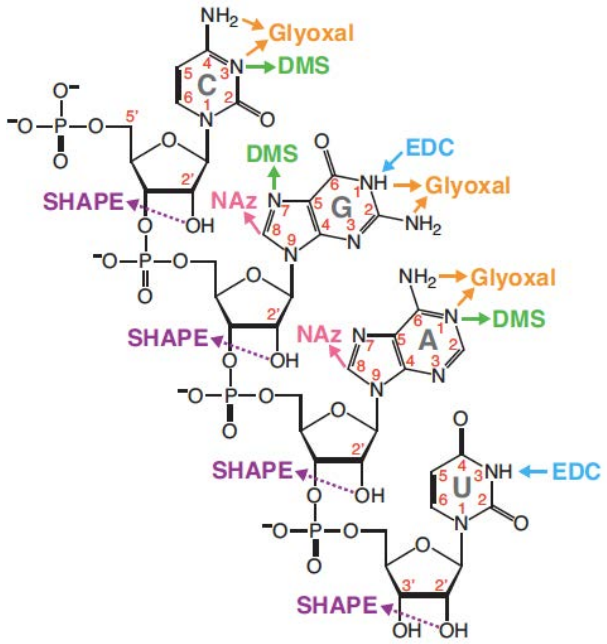
Assay	Probing Agent	Detection	In Vitro Probing	In Vivo Probing
FragSeq	P1 nuclease	single-stranded bases	X	
PARS	RNase V1 and S1 nuclease	paired and single-stranded regions	X	
SHAPE-seq	1M7	single-stranded bases	X	
mod-seq	DMS	unpaired A & C		X
DMS-seq	DMS	unpaired A & C	X	X
Structure-seq	DMS	unpaired 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

RNA structure: MaP, SHAPE, SHAPE-MaP, RING-MaP, Mod, CRIS etc...

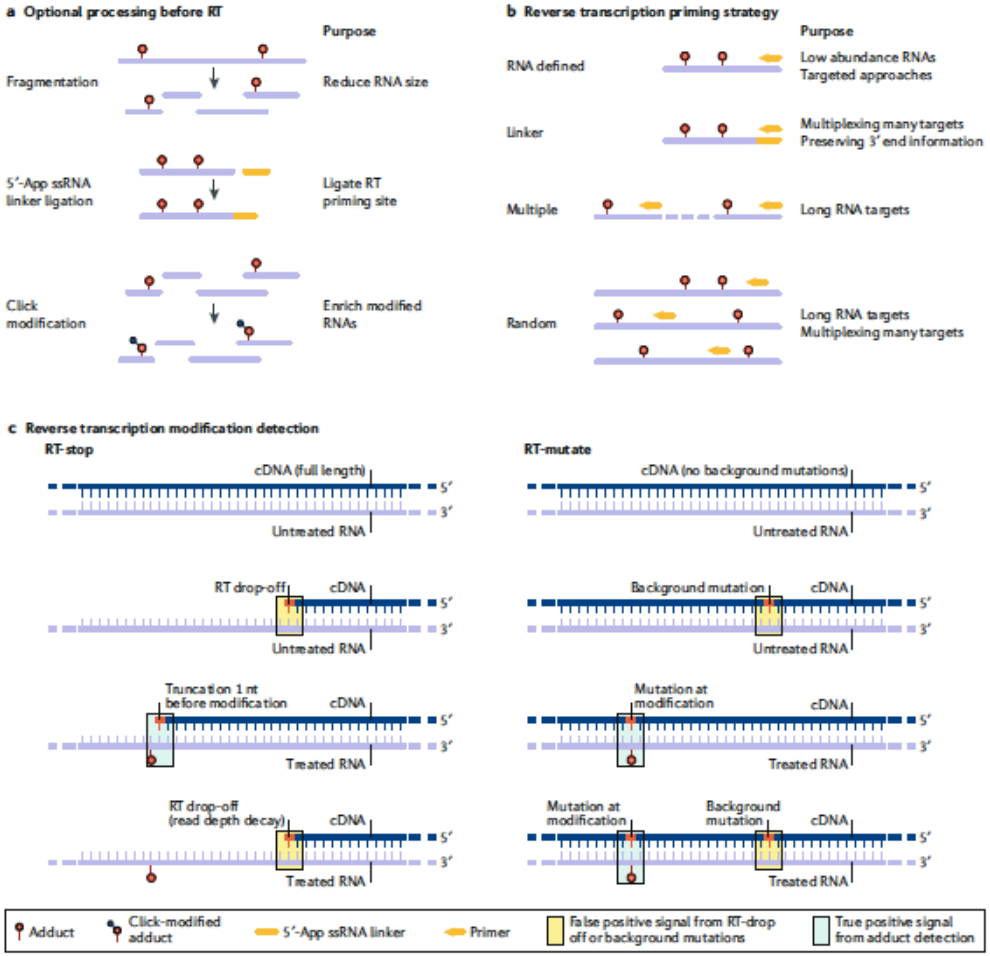


	Probe	Primary modification sites
SHAPE	*N*-methylisatoic anhydride (NMIA)	2′OH of all nts
	1-methyl-7-nitroisatoic anhydride (1M7)	2′OH of all nts
	1-methyl-6-nitroisatoic anhydride (1M6)	2′OH of all nts
	Benzoyl cyanide (BzCN)	2′OH of all nts
	2-methylnicotinic acid imidazolide (NAI)	2′OH of all nts
	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

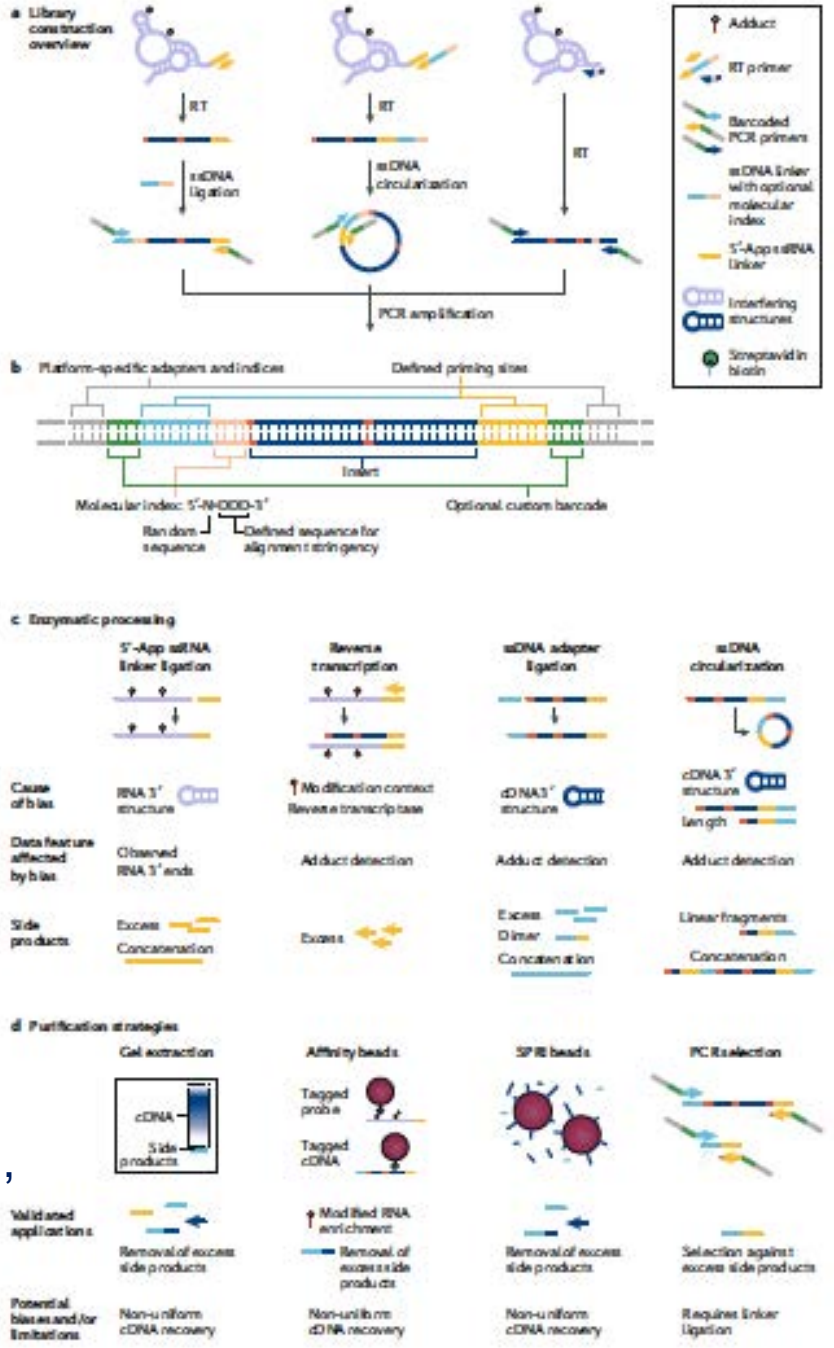


Mitchel III et al, CurrOpStructBiol, 2019
Strobel et al, NatRevGenet, 2018

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

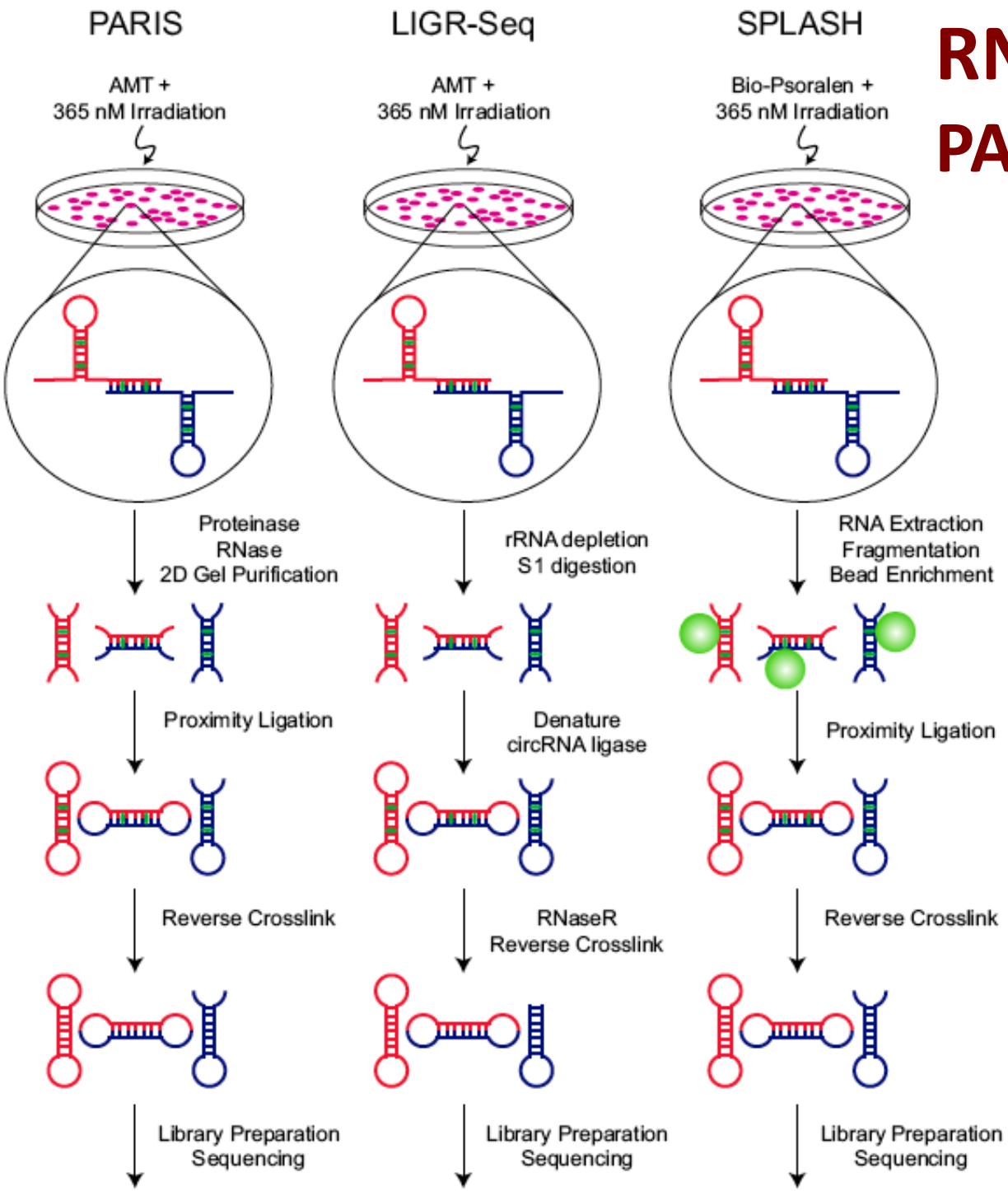


RNA structure



Strobel et al, NatRevGenet, 2018

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



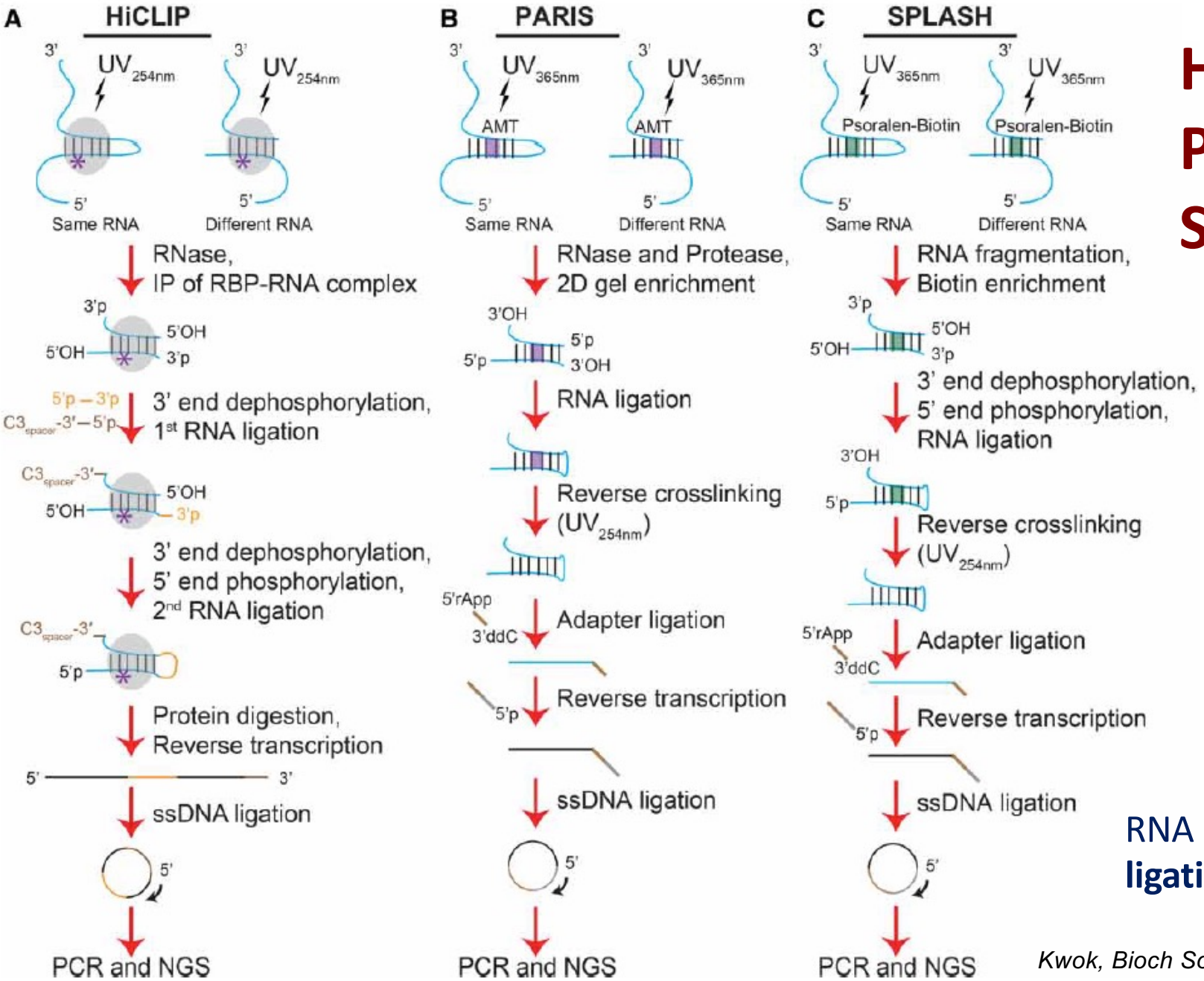
RNA structure
PARIS, SPLASH, LIGR

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

[AMT = psoralen derivative 4'-aminomethyltrioxalen]

Graveley, Mol Cell, 2016

RNA structure RNA-protein interactions

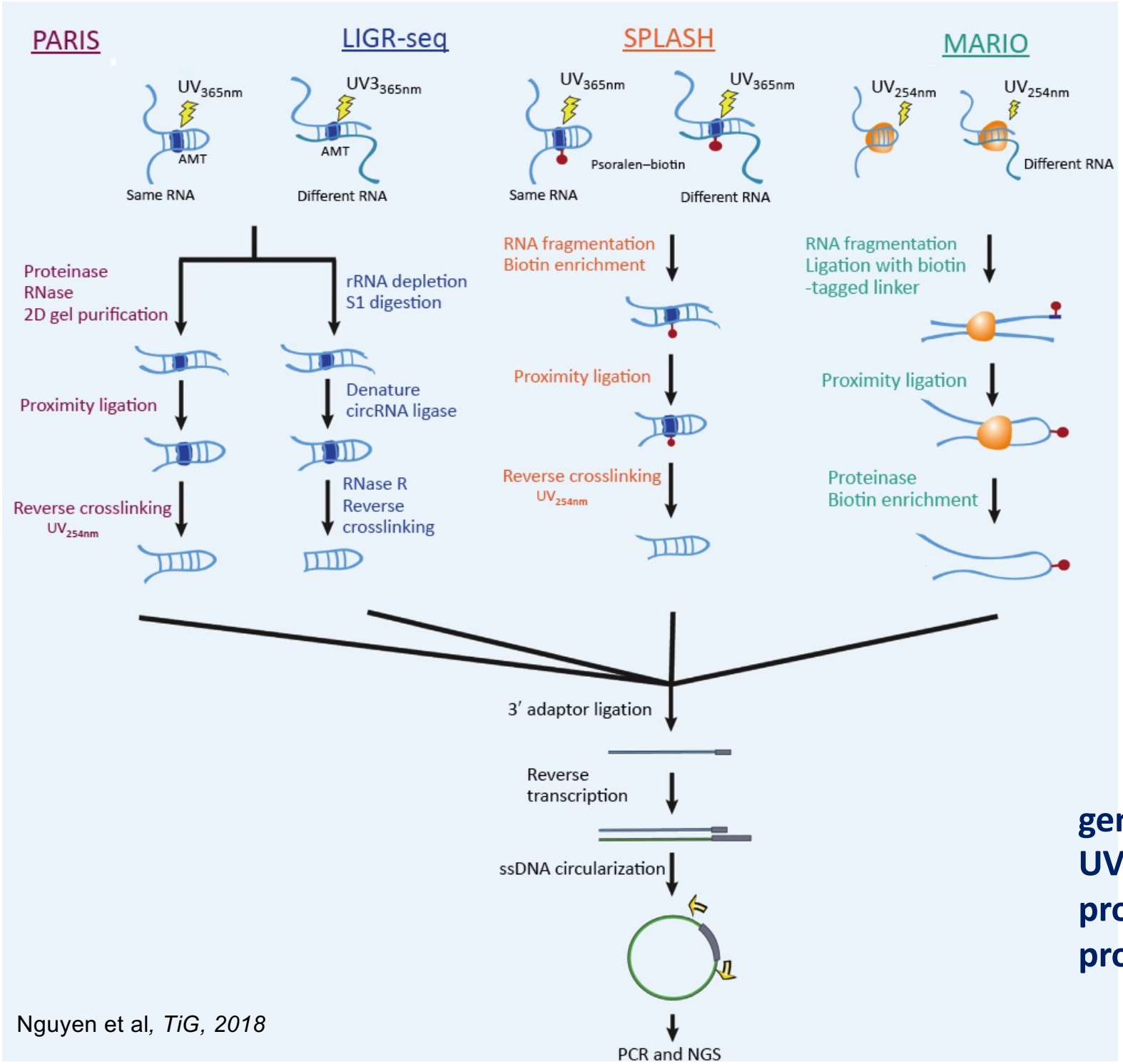


HiCLIP
PARIS
SPLASH

RNA **proximity ligation**

Kwok, Bioch Society Trans, 2015

RNA structure



Nguyen et al, *TiG, 2018*

genomewide
UV crosslink
protein-mediated
proximity ligation