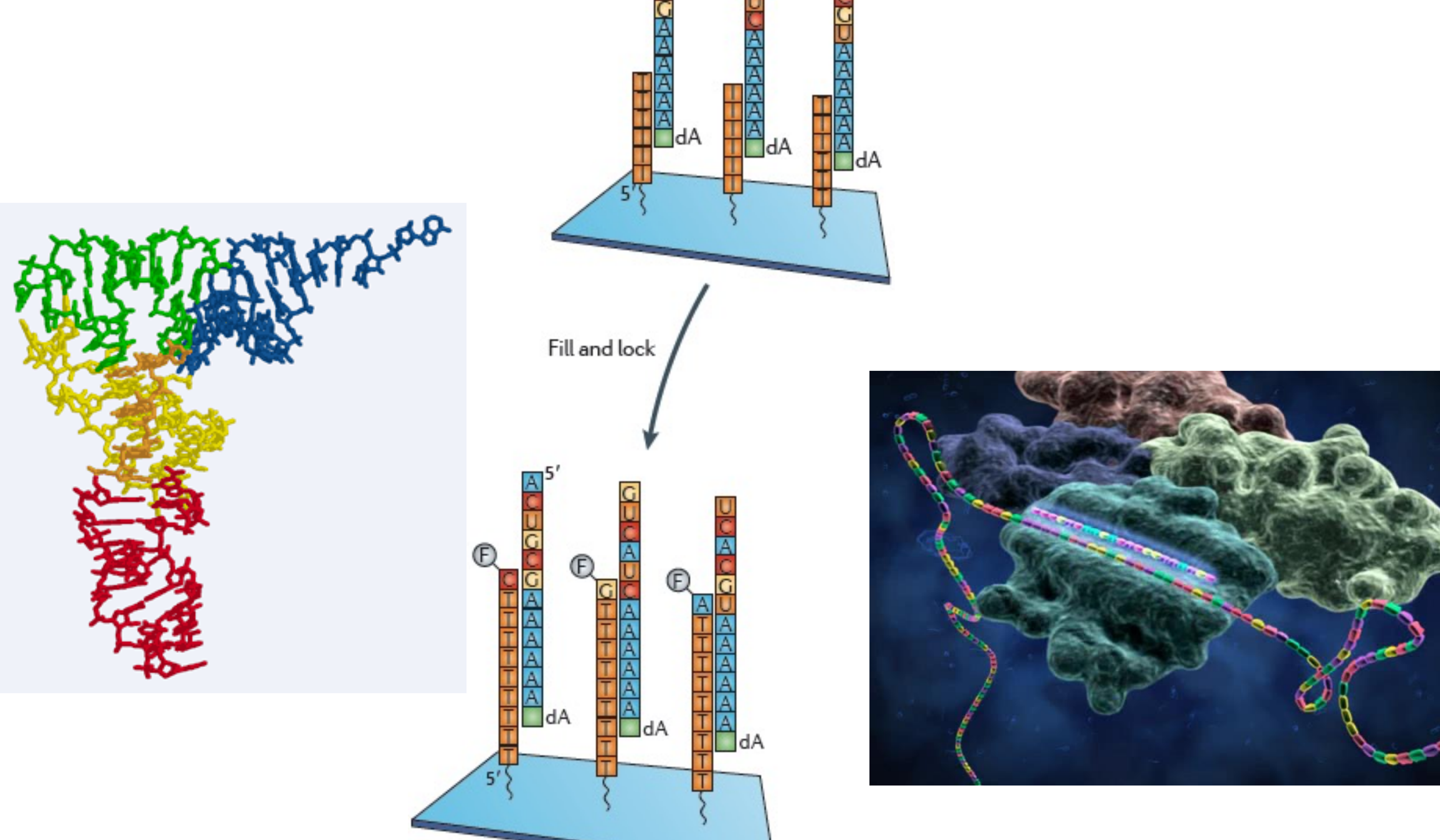
GLOBAL ANALYSES of RNAs and RNPs



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 and sequencing, indirectly reveals unknown ncRNAs**

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

- **ChIRP – Chromatin isolation by RNA Purification (+RNA-Seq)**
- **ChART - Capture Hybridization Analysis of RNA targets (+RNA-Seq)**

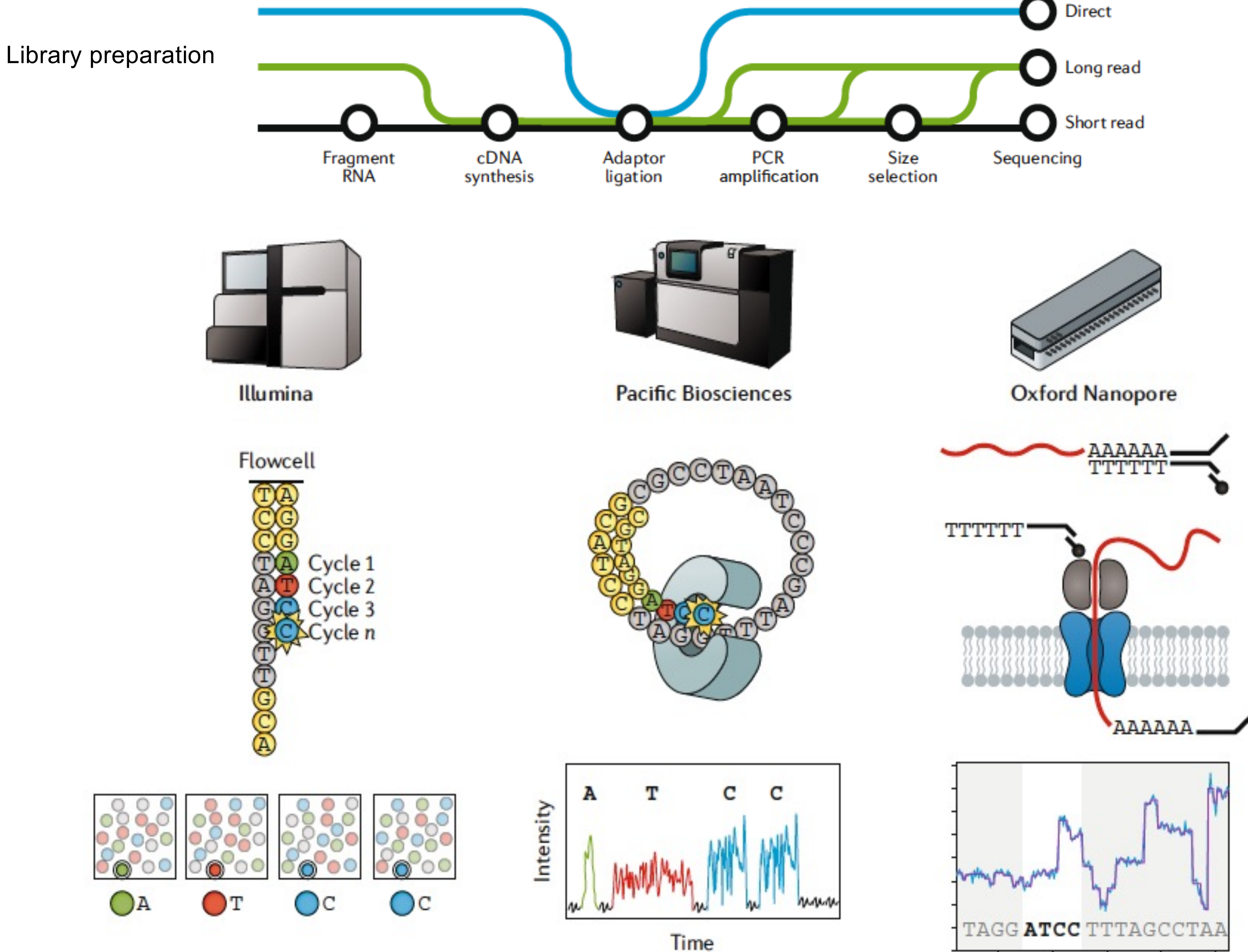
biotinylated oligonucleotides used to enrich for DNA sequences associated with a particular RNA

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

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

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

RNA-seq technologies



Stark et al, Nat Rev Genet, 2019

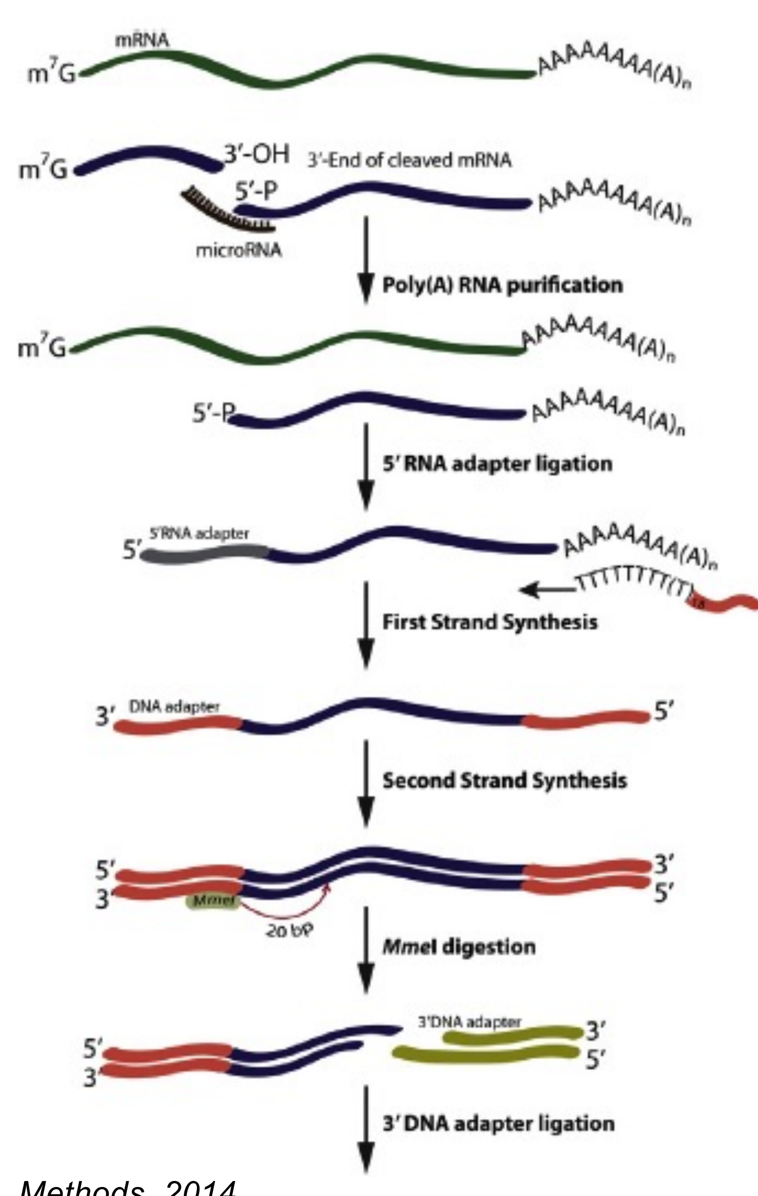
RNA-seq technologies

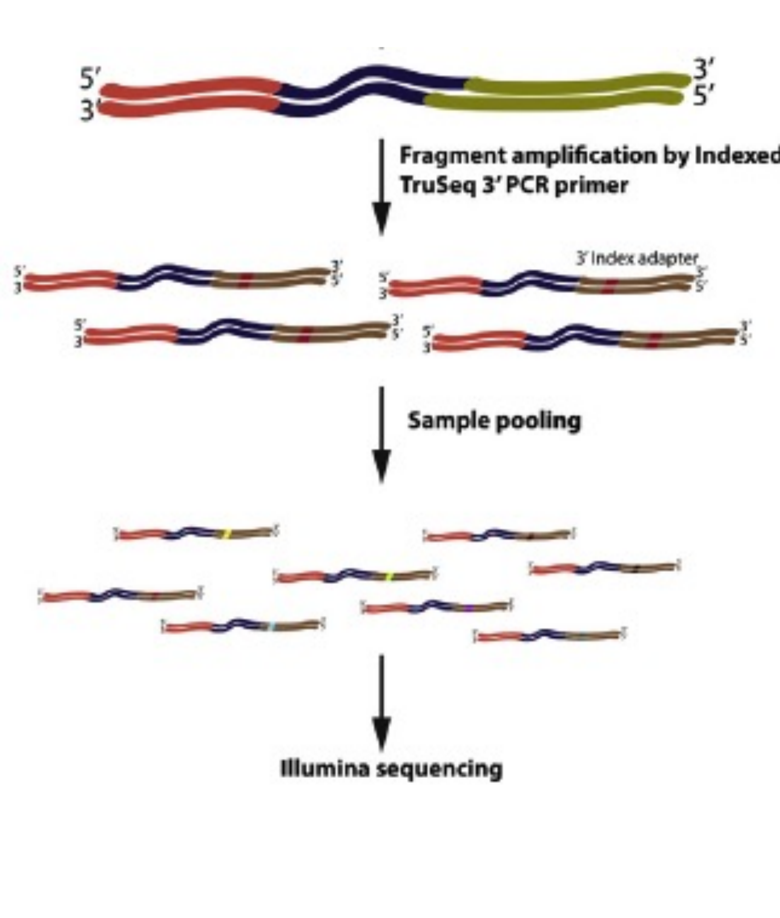
Sequencing technology	Platform	Advantages	Disadvantages	Key applications
Short-read cDNA	Illumina, Ion Torrent	• Technology features very high throughput: currently 100–1,000 times more reads per run than long-read platforms • Biases and error profiles are well understood (homopolymers are still an issue for Ion Torrent) • A huge catalogue of compatible methods and computational workflows are available • Analysis works with degraded RNA	• Sample preparation includes reverse transcription, PCR and size selection adding biases to all methods • Isoform detection and quantitation can be limited • Transcript discovery methods require a de novo transcriptome alignment and/or assembly step	Nearly all RNA-seq methods have been developed for short-read cDNA sequencing: DGE, WTA, small RNA, single-cell, spatialomics, nascent RNA, translatome, structural and RNA–protein interaction analysis, and more are all possible
Long-read cDNA	PacBio, ONT	• Long reads of 1–50 kb capture many full-length transcripts • Computational methods for de novo transcriptome analysis are simplified	• Technology features low-to-medium throughput: currently only 500,000 to 10 million reads per run • Sample preparation includes reverse transcription, PCR and size selection (for some protocols), adding biases to many methods • Degraded RNA analysis is not recommended	Sequencing is particularly suited to isoform discovery, de novo transcriptome analysis, fusion transcript discovery, and MHC, HLA or other complex transcript analysis
Long-read RNA	ONT	• Long reads of 1–50 kb capture many full-length transcripts • Computational methods for de novo transcriptome analysis are simplified • Sample preparation does not require reverse transcription or PCR-reducing biases • RNA base modifications can be detected • Poly(A) tail lengths can be directly estimated from single-molecule sequencing	• Technology features low throughput: currently only 500,000 to 1 million reads per run • Sample preparation and sequencing biases are not well understood • Degraded RNA analysis is not recommended	• Sequencing is particularly suited to isoform discovery, de novo transcriptome analysis, fusion transcript discovery, and MHC, HLA or other complex transcript analysis • Ribonucelotide modifications can be detected

Stark et al, Nat Rev Genet, 2019

PARE: Parallel Analysis of RNA End

mRNA DEGRADOME RNA-seq

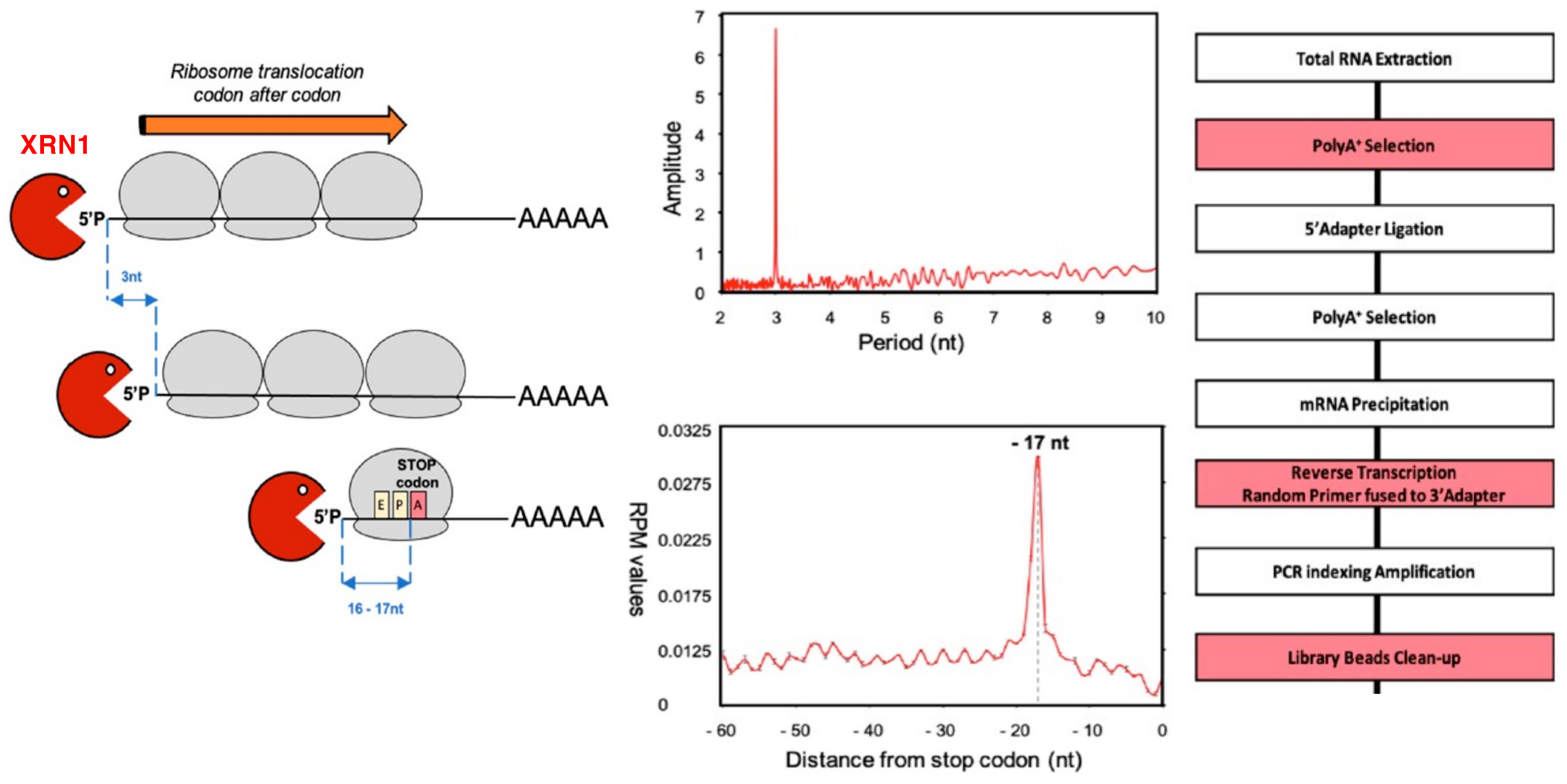




Zhai et al., Methods, 2014

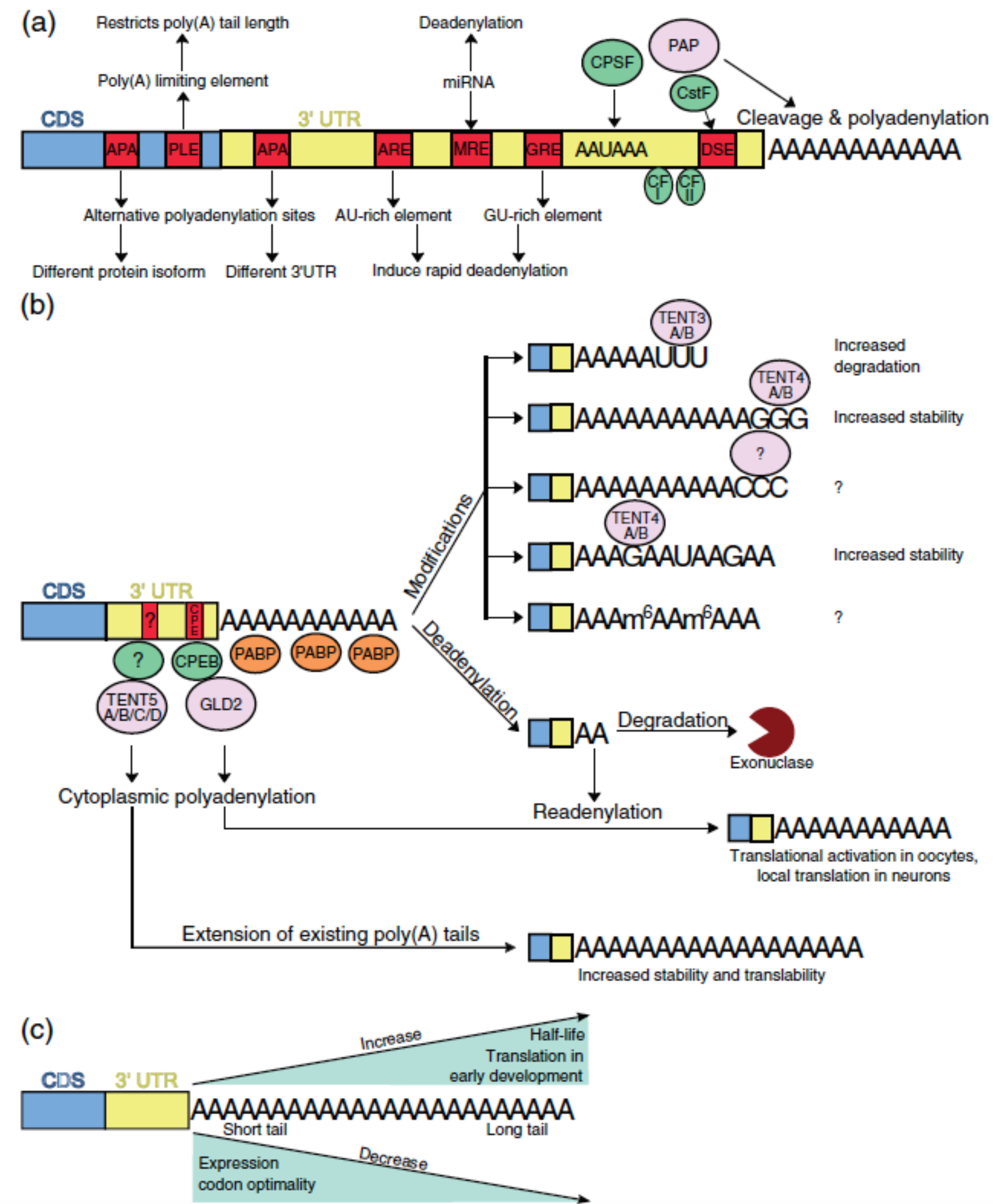
GMUCT: Genome-wide Mapping of Uncapped Transcripts

Analysis of Co-Translational mRNA Decay (CTRD)



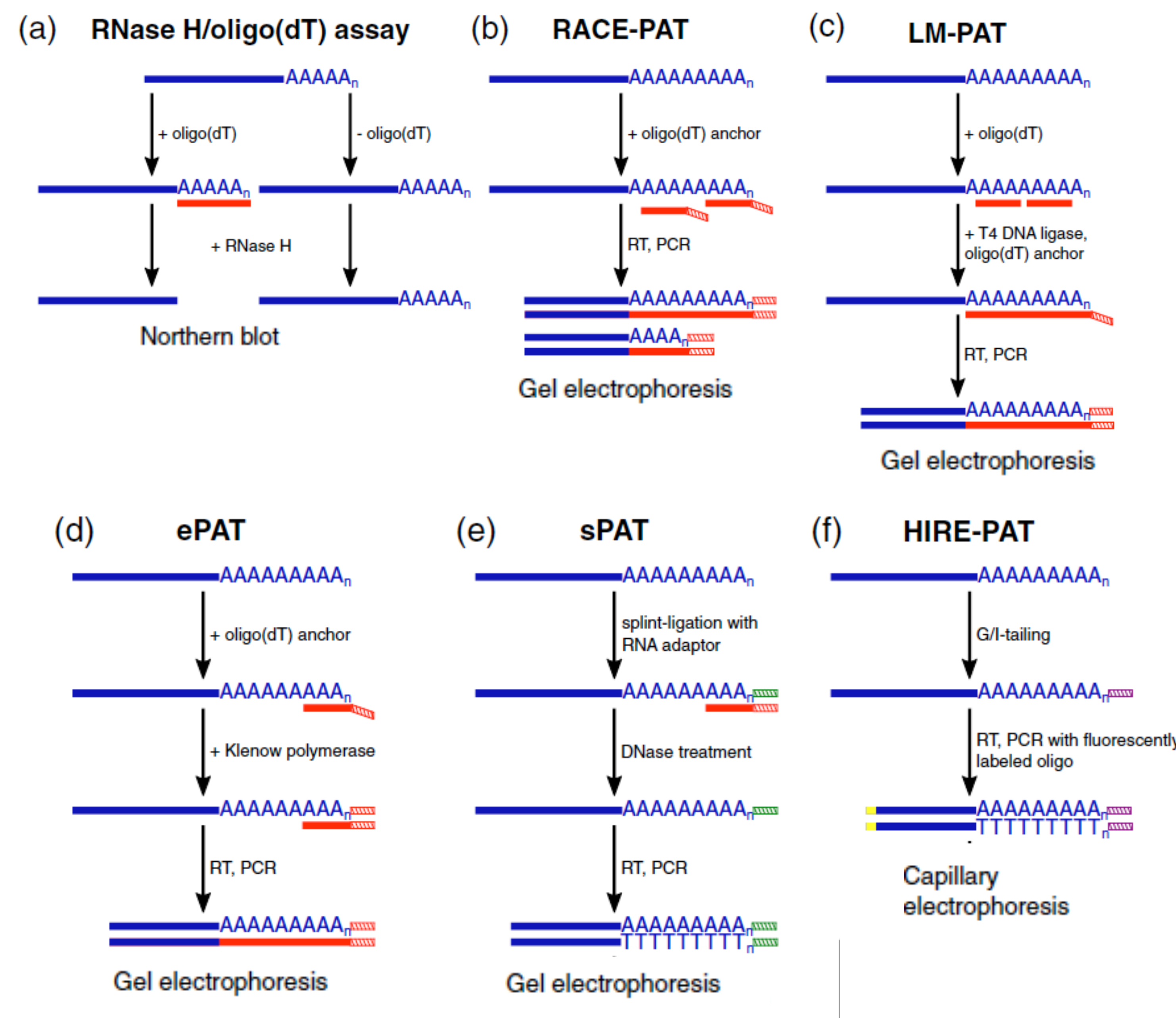
Carpentier et al., Plants, 2021

3’ end and poly(A) tail analyses



Brouze et al, WIREsRNA, 2022

Poly(A) tail analyses, classical methods



Brouze et al, WIREsRNA, 2022

DRS: Direct RNA sequencing of Poly(A) sites

poly(A) sites

poly(A) sites

PAT-seq: poly(A) tag sequencing

Sherstnev et al., Nat Str Mol Biol, 2012; Ma et al., Methods 2013

Total RNA > 200 nt, rRNA-depleted

AAA..

Messenger RNA   short ncRNA

3′ adaptor ligation

AAA.. B   B

Partial digestion with RNase T1

AAA.. B   B

Pull-down with streptavidin
5′ end phosphorylation

AAA.. B   B

Gel purification
(500 – 1000 nt)

AAA.. B

5′ adaptor ligation

AAA.. B

RT, PCR,
and sequencing

Read 1 (51 nt)

AAA..
TTT..

Read 2
(251 nt)

Method A

AAAAAAAA

Poly(A) enrichment, reverse transcriptase, with anchored oligo-dT18 attached to one sequencing adapter ( )

AAAAAAAA
TTTTTTTT

Second strand synthesis

AAAAAAAA
TTTTTTTT

Restriction digestion (4 base cutter), ligation with second sequencing adapter ( )

AAAAAAAA
TTTTTTTT

Amplify, purify, submit for sequencing

AAAAAAAA
TTTTTTTT

Method B

AAAAAAAA

Fragment, Poly(A) enrichment

AAAAAAAA

Reverse transcriptase, with anchored oligo-dT18 attached to one sequencing adapter ( ), and other sequencing adapter attached to a strand-switching primer ( )

AAAAAAAA
TTTTTTTT

Amplify, purify, submit for sequencing

Size-enrichment (AMPure bead method)

AAAAAAAA
TTTTTTTT

AAAAAAAA
TTTTTTTT

Method B1

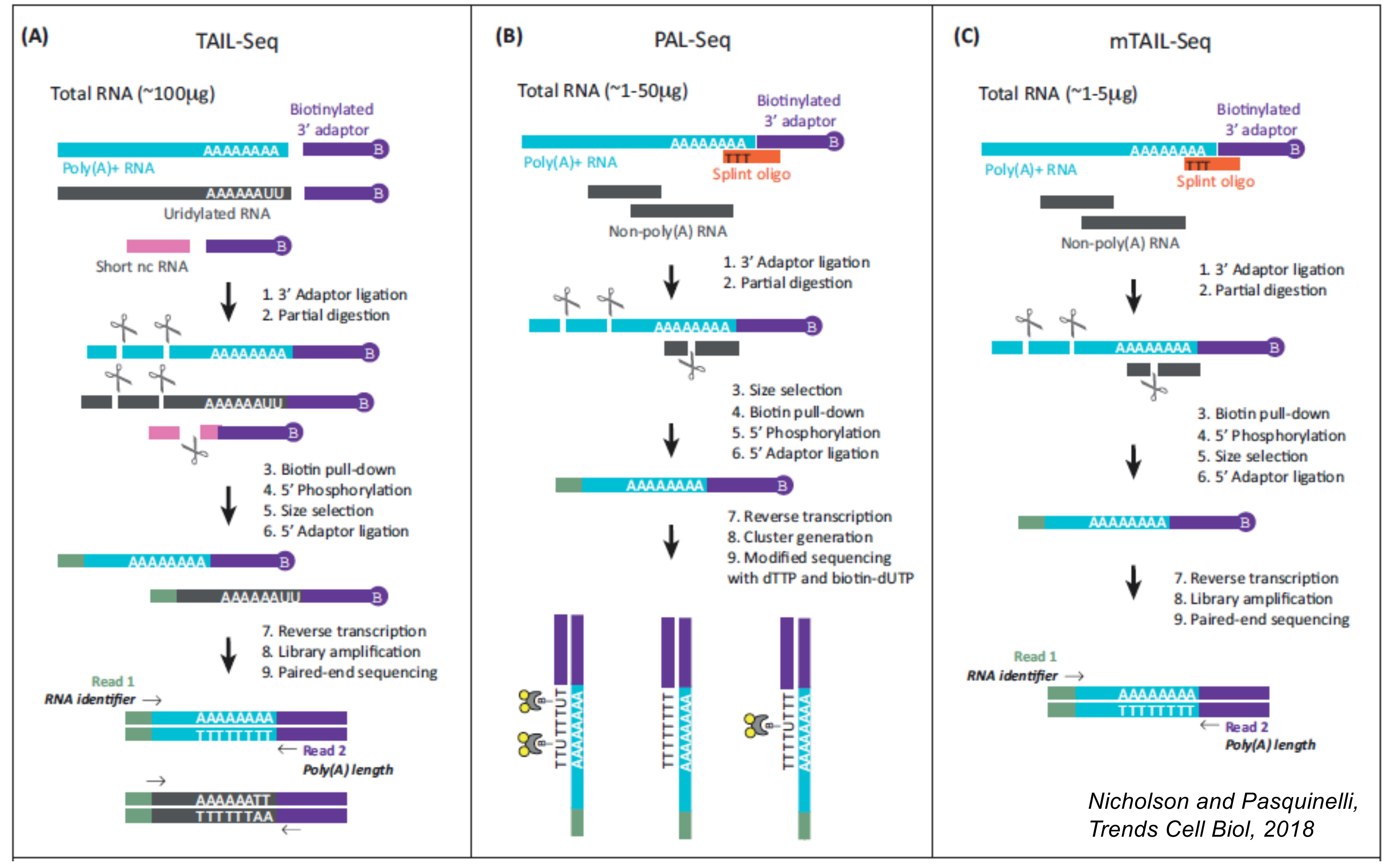
Amplify, purify, submit for sequencing

AAAAAAAA
TTTTTTTT

Method B2

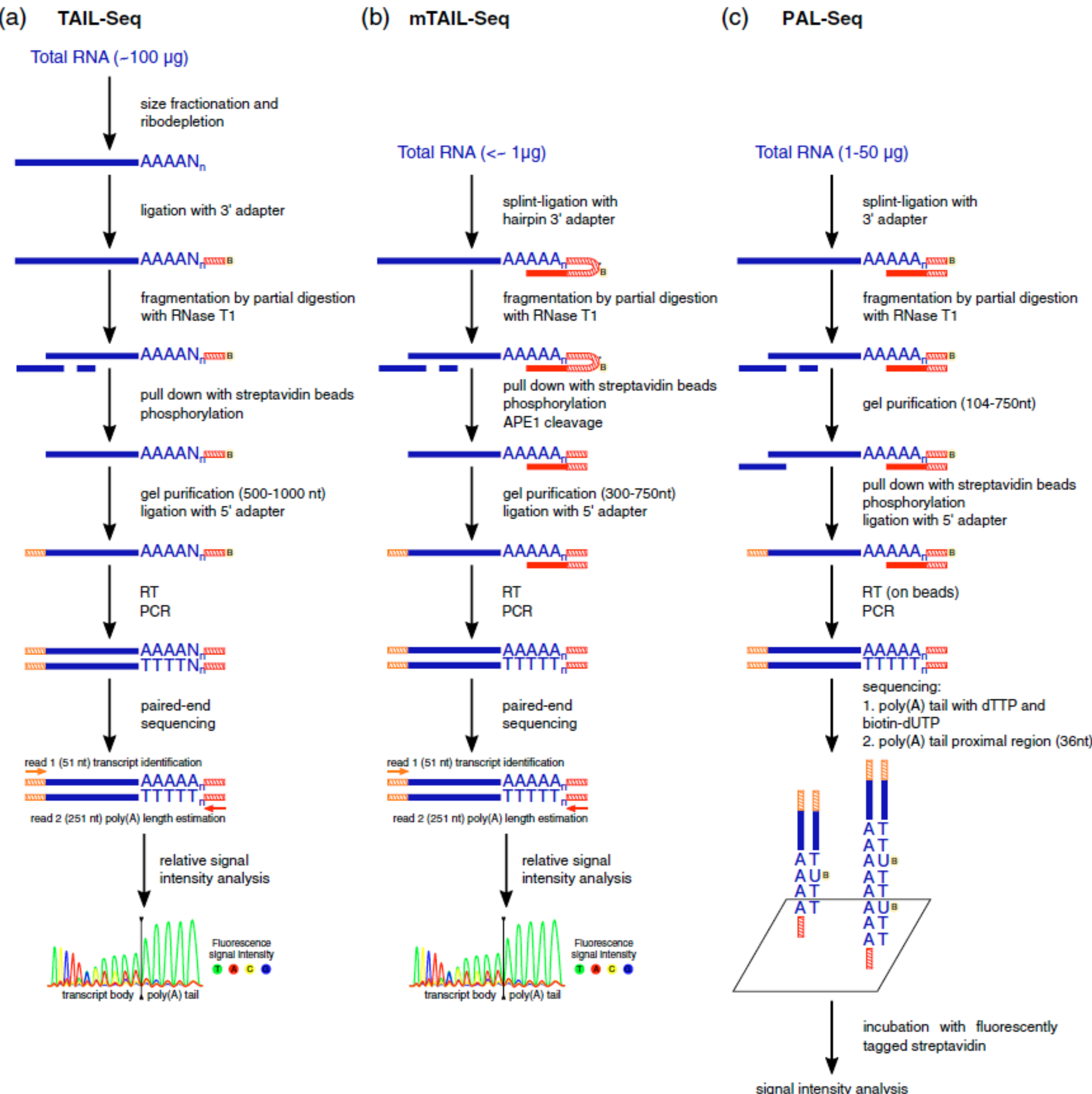
TAIL-seq: RNA 3' end sequencing

Poly(A) tail length and 3' end modifications (e.g. U-tailing)



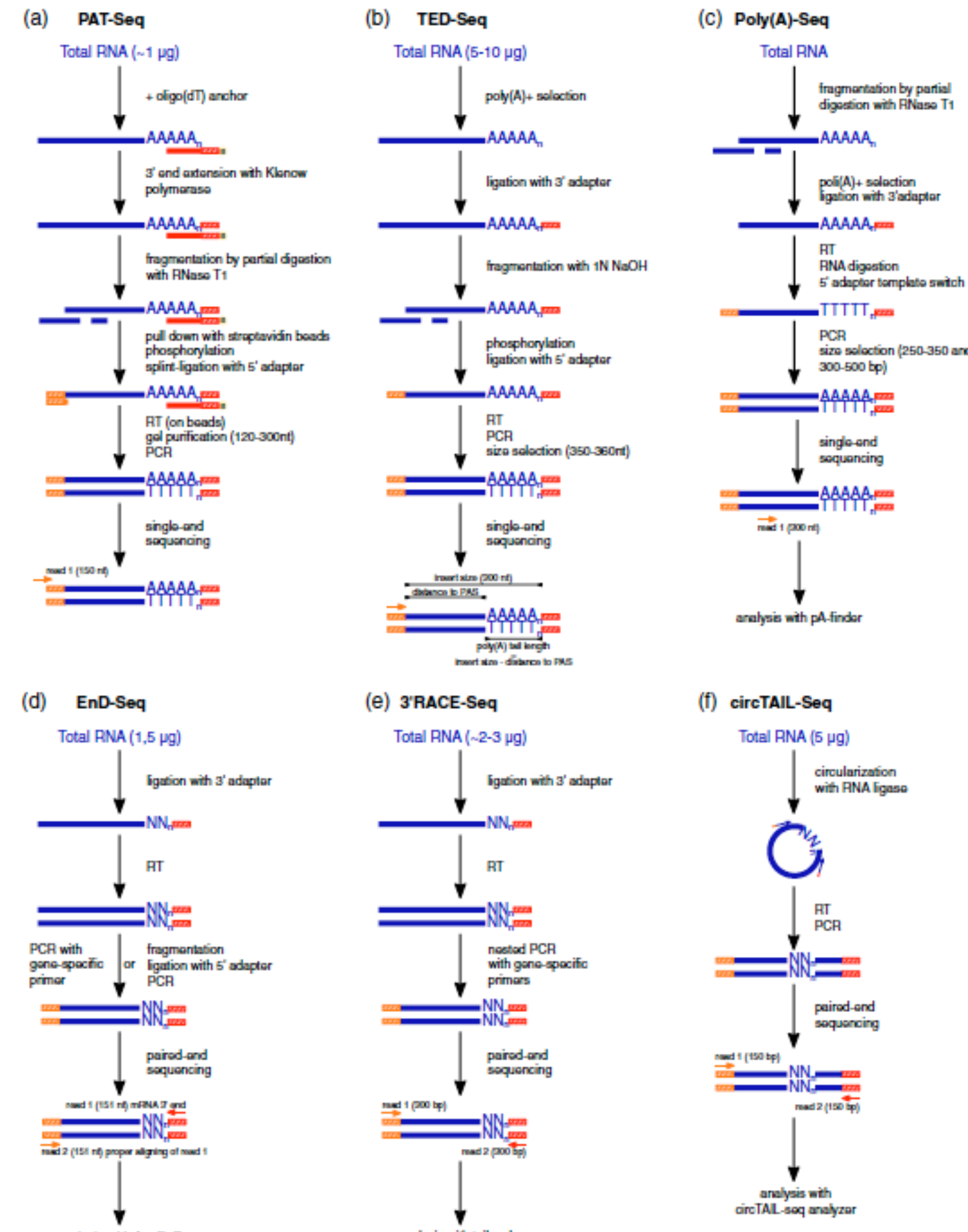
Nicholson and Pasquinelli, Trends Cell Biol, 2018

Poly(A) tail analysis



Brouze et al, WIREsRNA, 2022

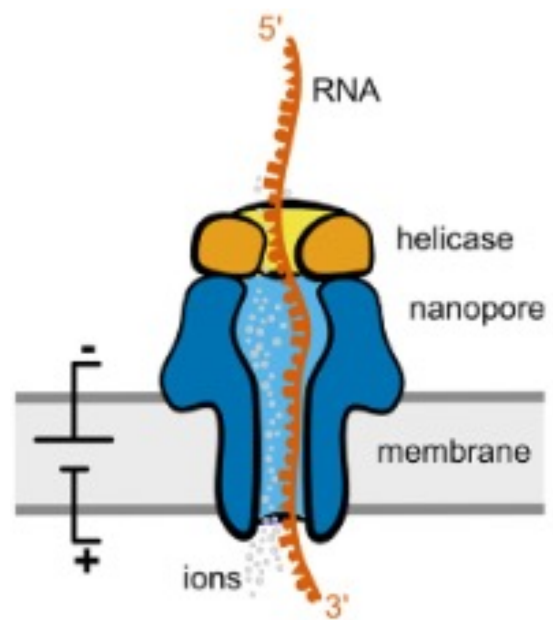
3’ end RNA analysis

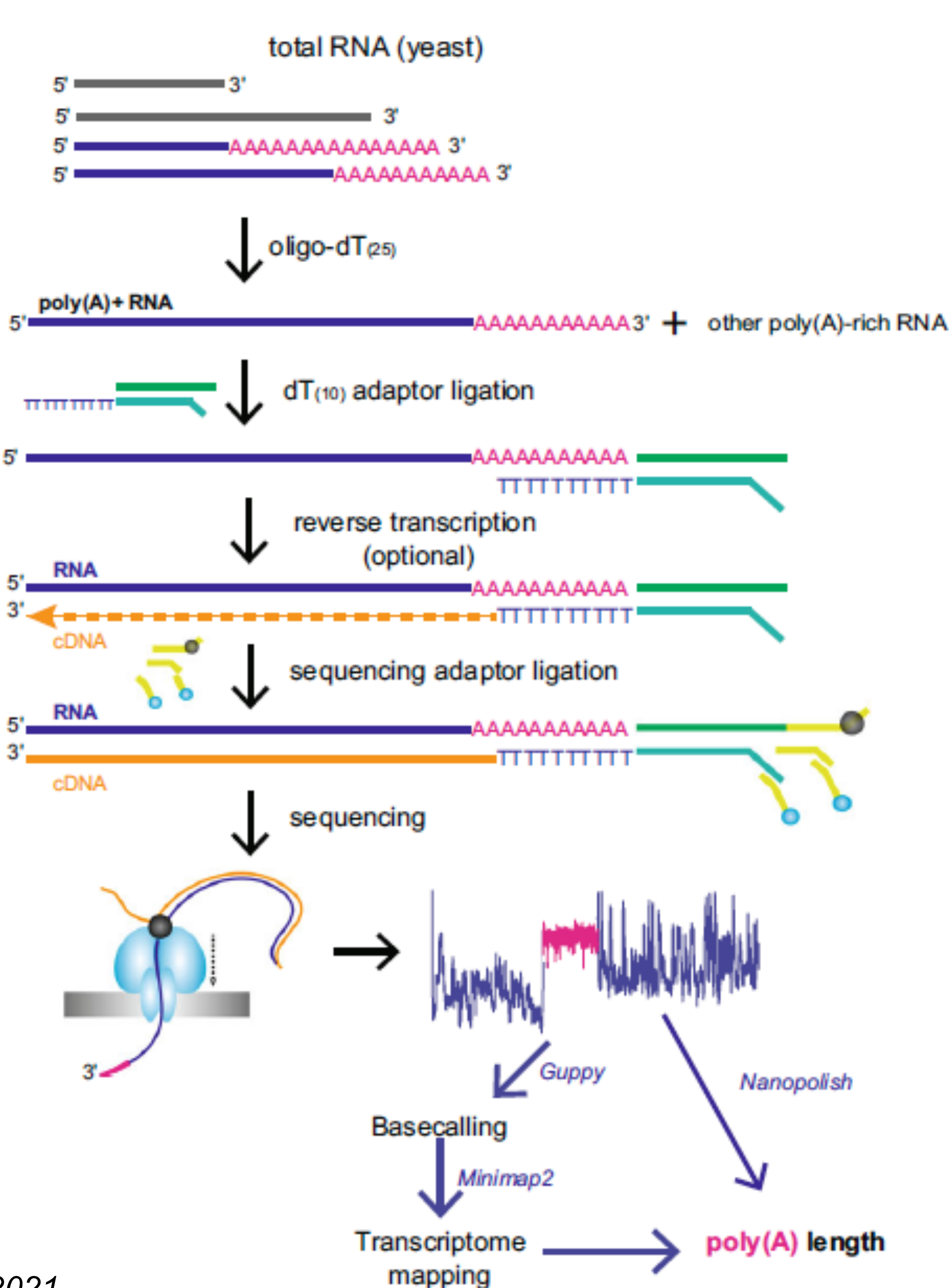


Brouze et al, WIREsRNA, 2022

Nanopore long read sequencing

DNA and RNA -seq
DRS or cDNA-based
Poly(A) tail analysis
RNA modification mapping



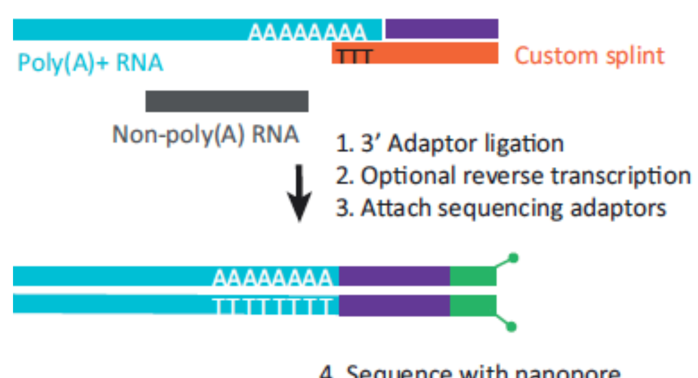


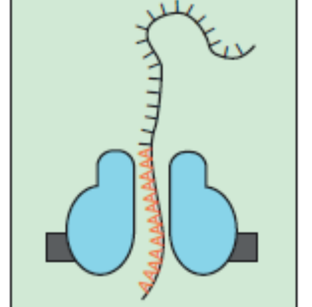
- **For polyA$^+$ RNA**
- **For nonpolyadenylated RNA addition of poly(A) or poly(I) is required**
- **Lower deepth than NGS**

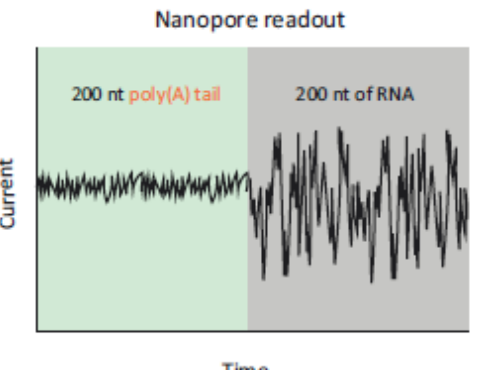
Ray and Hasselberth, Front Genet 2022; Tudek et al, Nat Comm, 2021

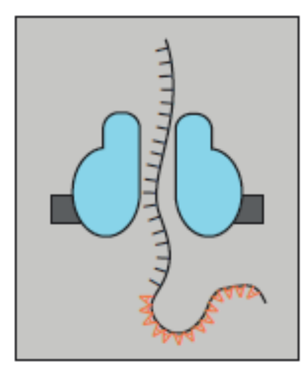
Nanopore

Poly(A) tail analyses

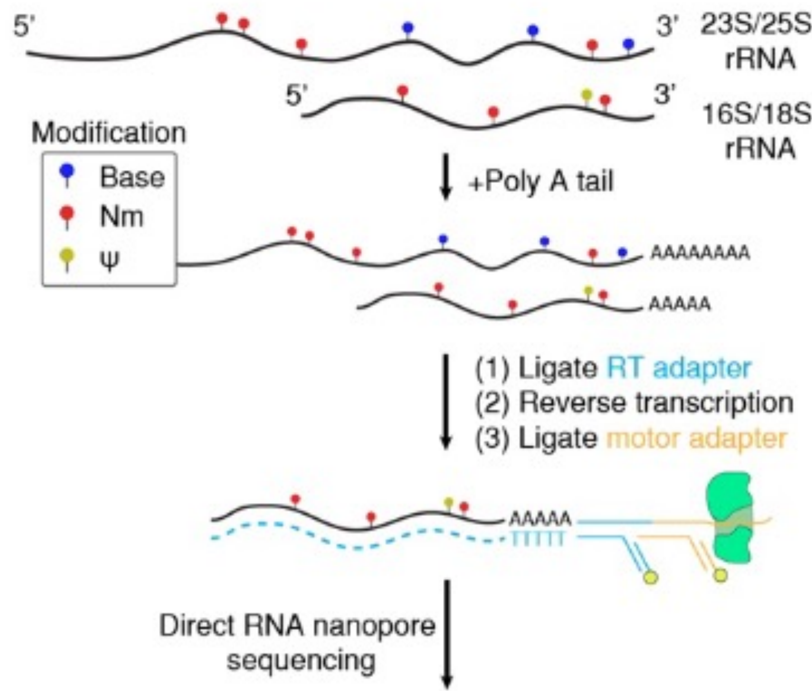


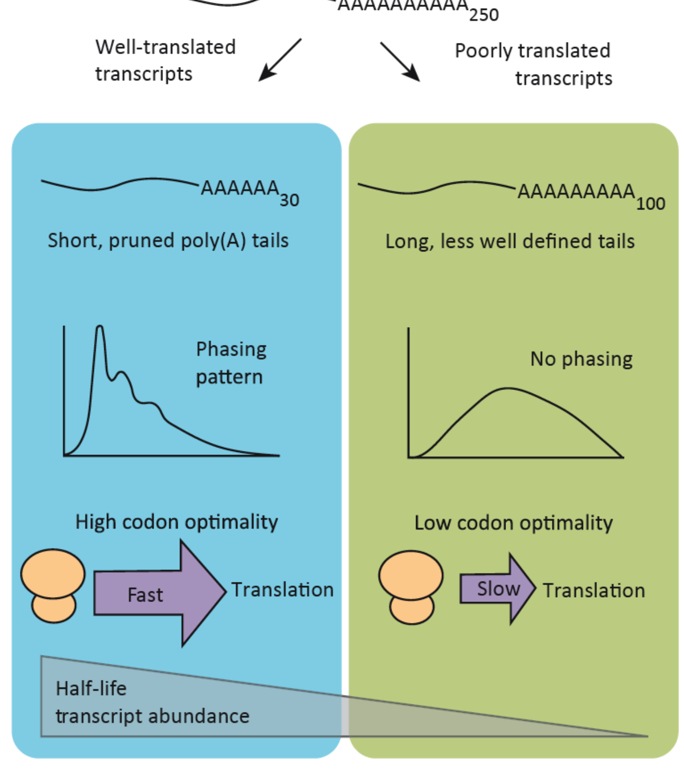






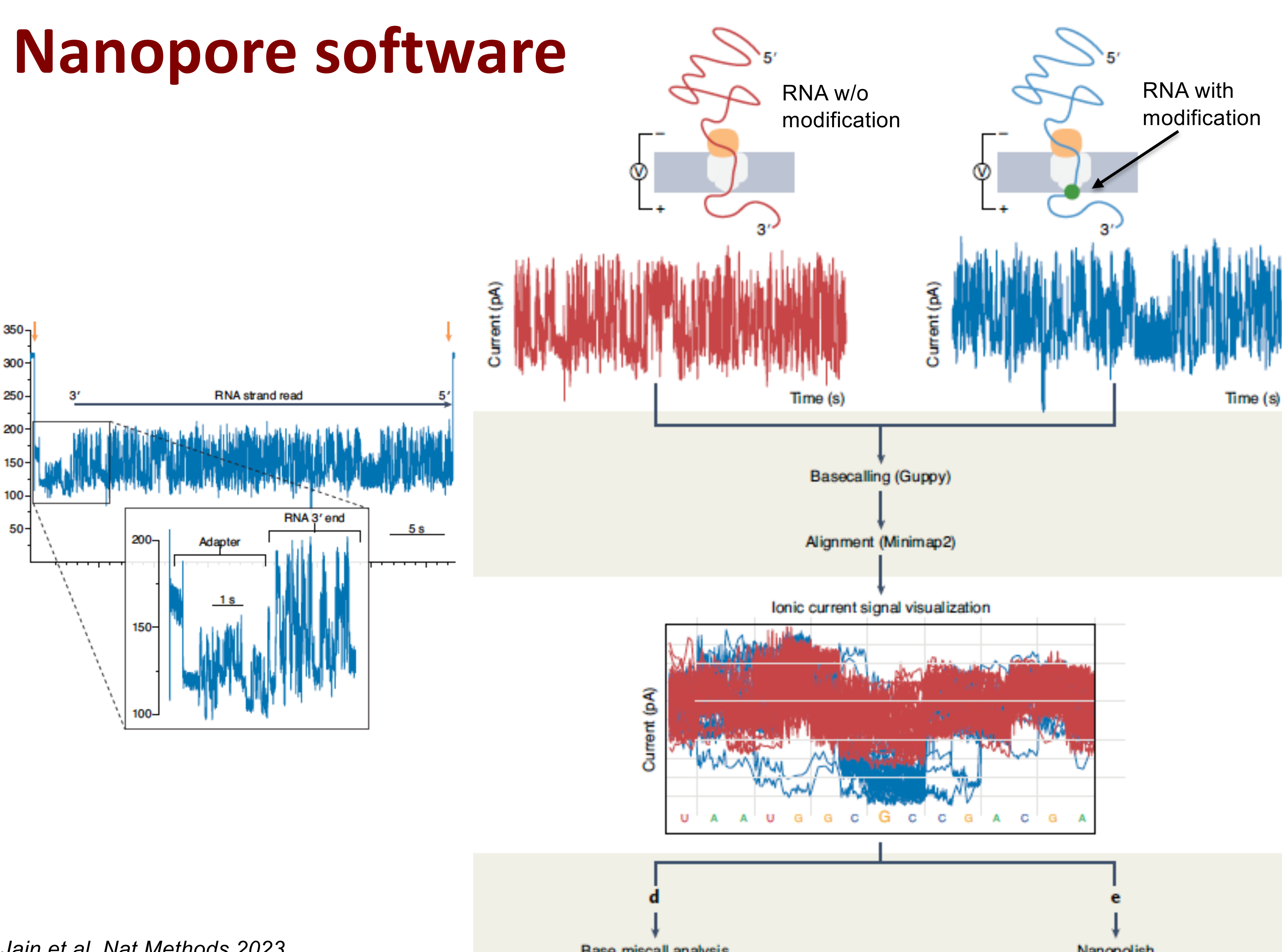
RNA modifications





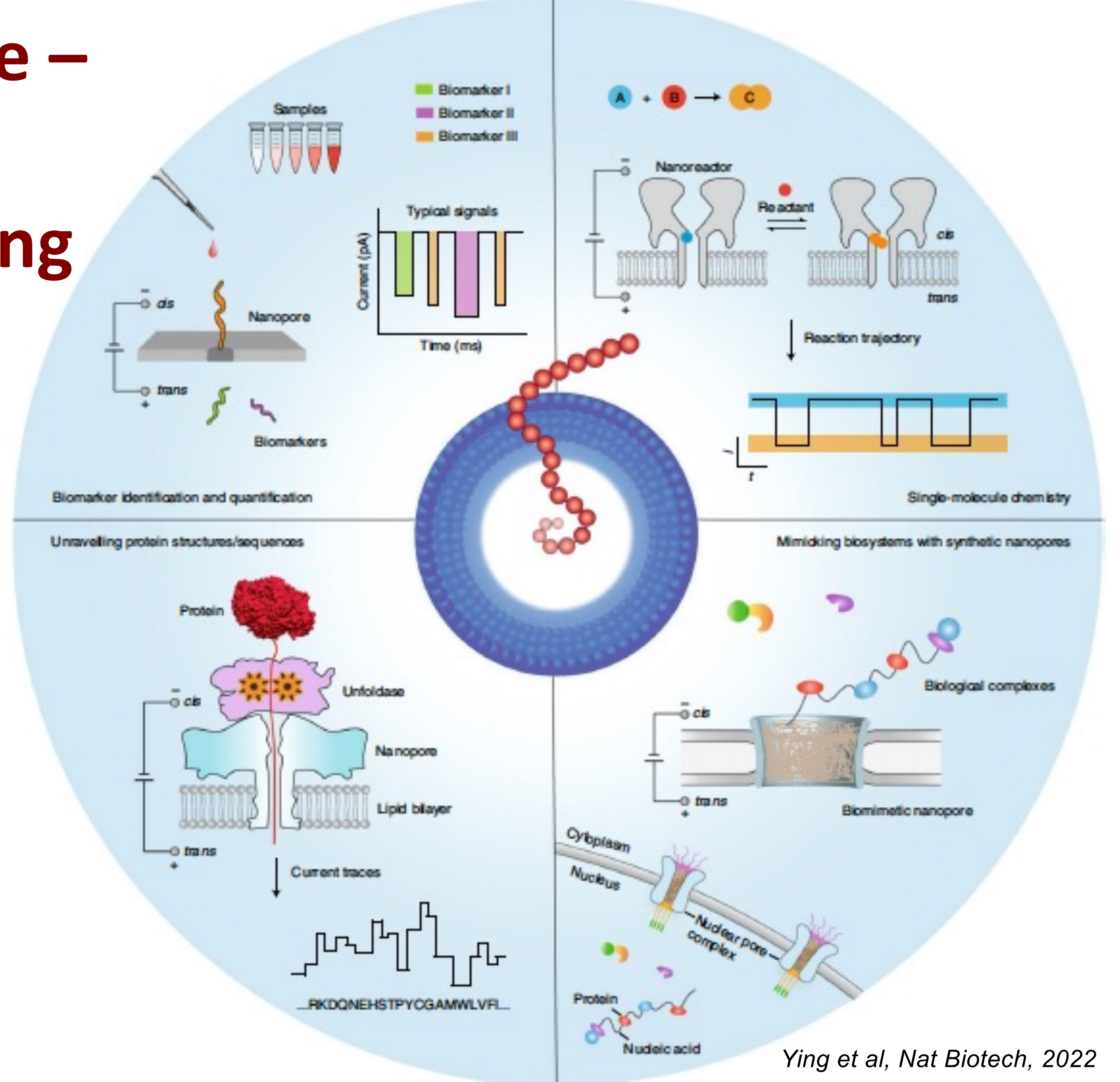
Nicholson and Pasquinelli, TiCB 2018

Nanopore software



Jain et al, Nat Methods 2023

Nanopore – beyond sequencing

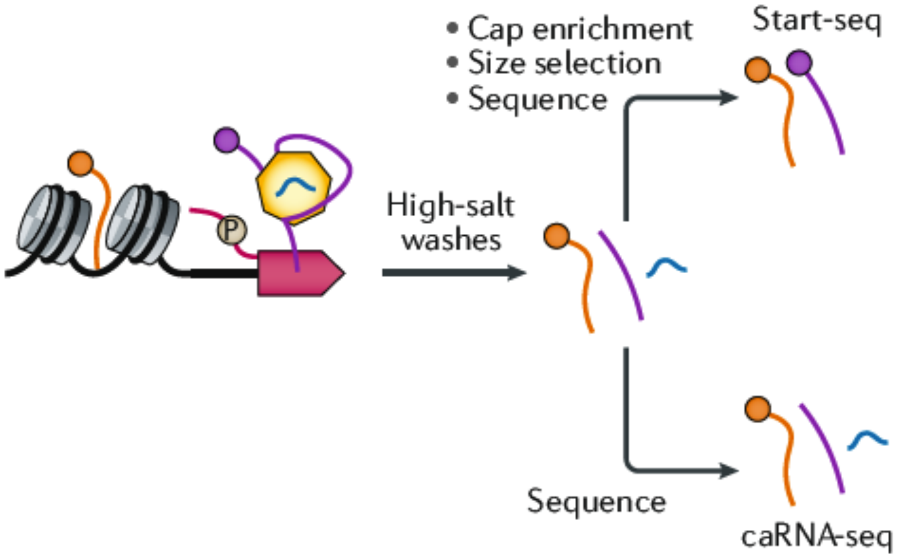


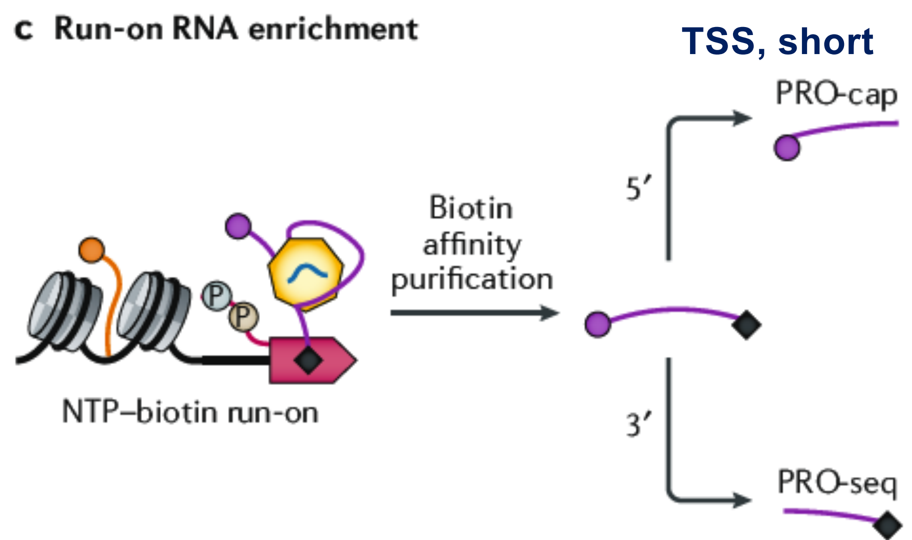
Ying et al, Nat Biotech, 2022

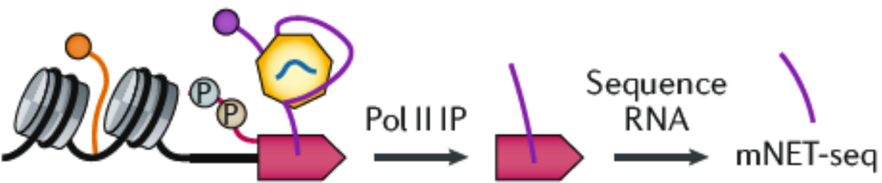
Nascent RNA analyses

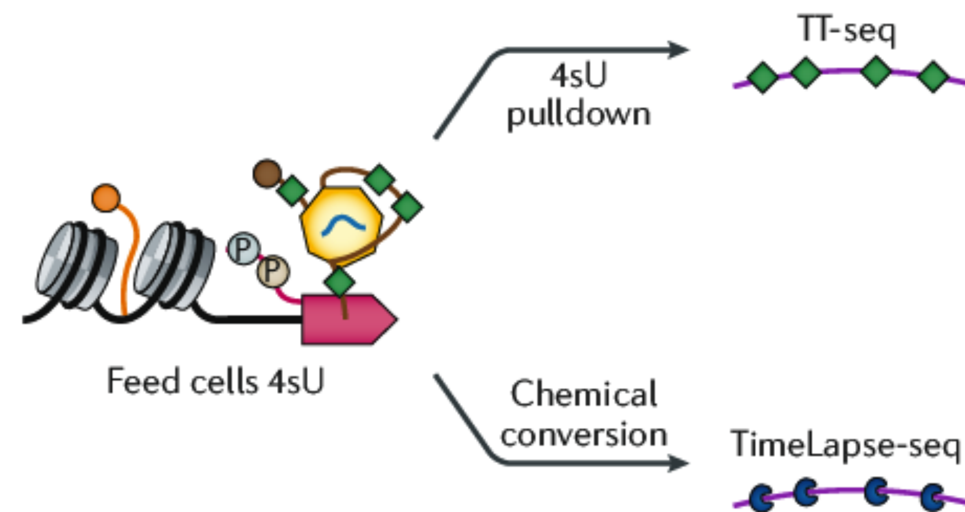
IP-based, formaldehyde crosslink

Purification of transcribed RNAs



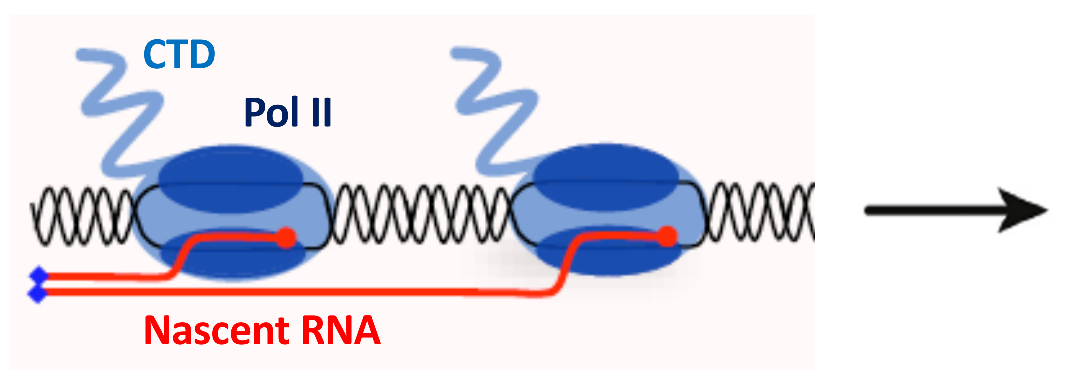






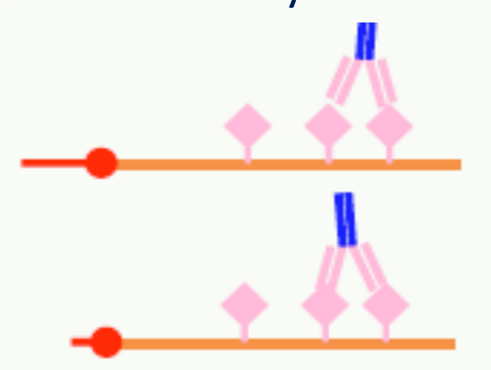
Wissink et al, Nat Rev Genet, 2019

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

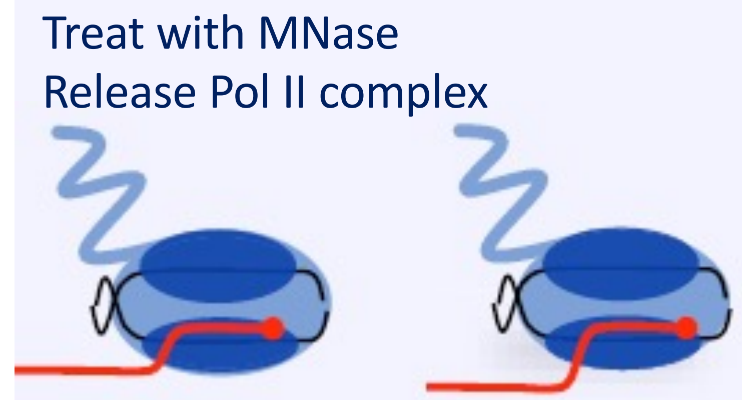


ChaRNA-seq

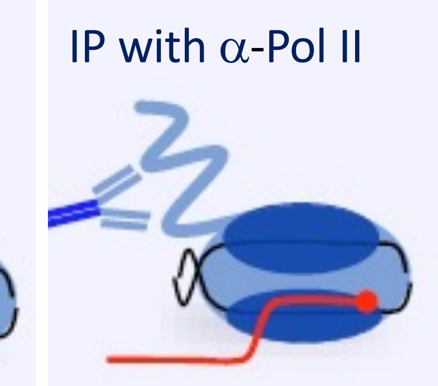
Prepare chromatin
Isolate chromatin-bound RNA
Deplete poly(A) and rRNA

NET-seq

Treat with MNase
Release Pol II complex

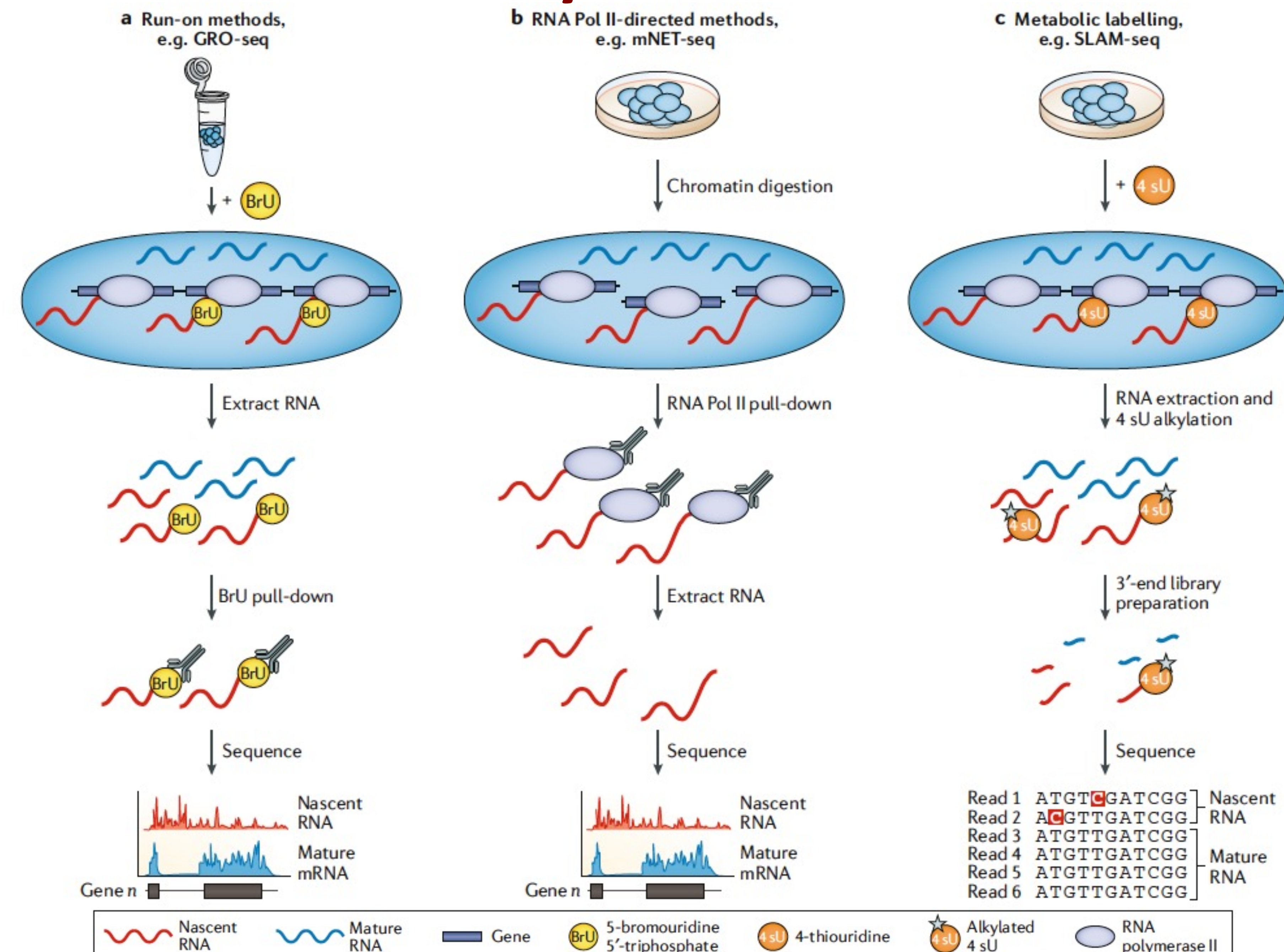


IP with α-Pol II



Liu et al, Plant Cell, 2023

Nascent RNA analyses



Stark 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

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]

Short-read and long-read sequencing methods for genome-wide characterization of nascent RNAs

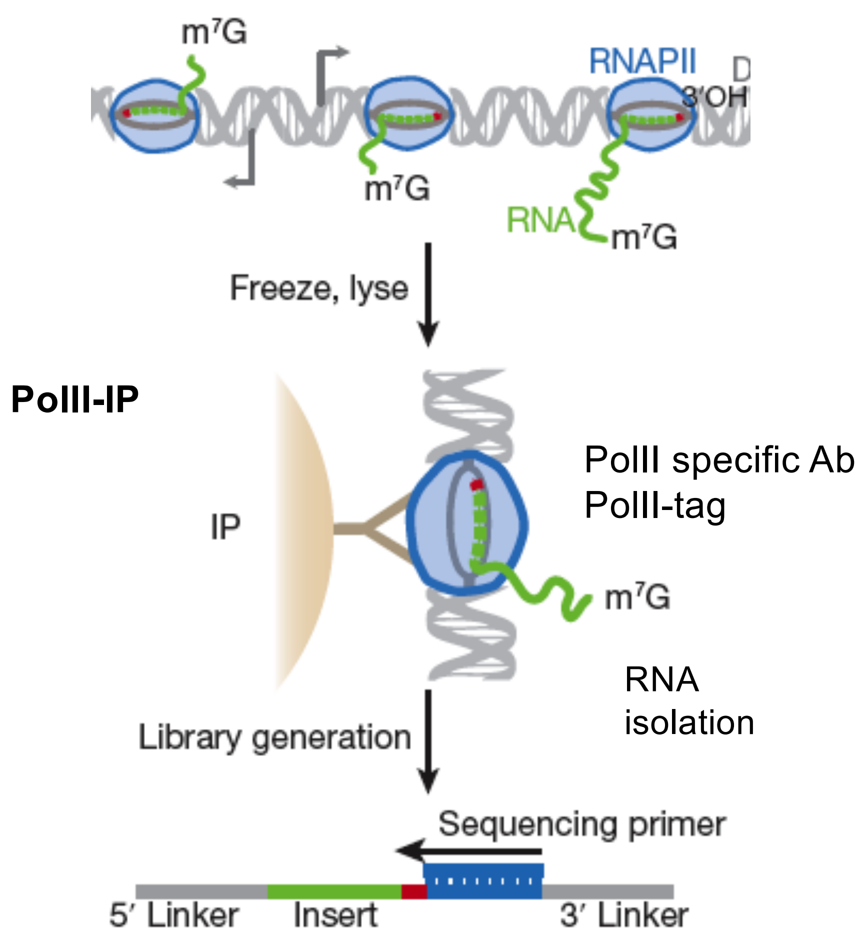
Elongation	Co-transcriptional splicing	Polyadenylation		Termination
GRO-seq	CB-RNA-seq	PAL-seq	DRS	GRO-seq
pNET-seq	Nano-COP	PAT-seq	PAIso-seq	pNET-seq
plaNET-seq	FLEP-seq	TAIL-seq	FLEP-seq	plaNET-seq
Nano-COP	POINT-nano	Poly(A)-seq	FLAM-seq	FLEP-seq
FLEP-seq				
POINT-nano				

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

Analysis of Nascent Transcripts

NET-seq

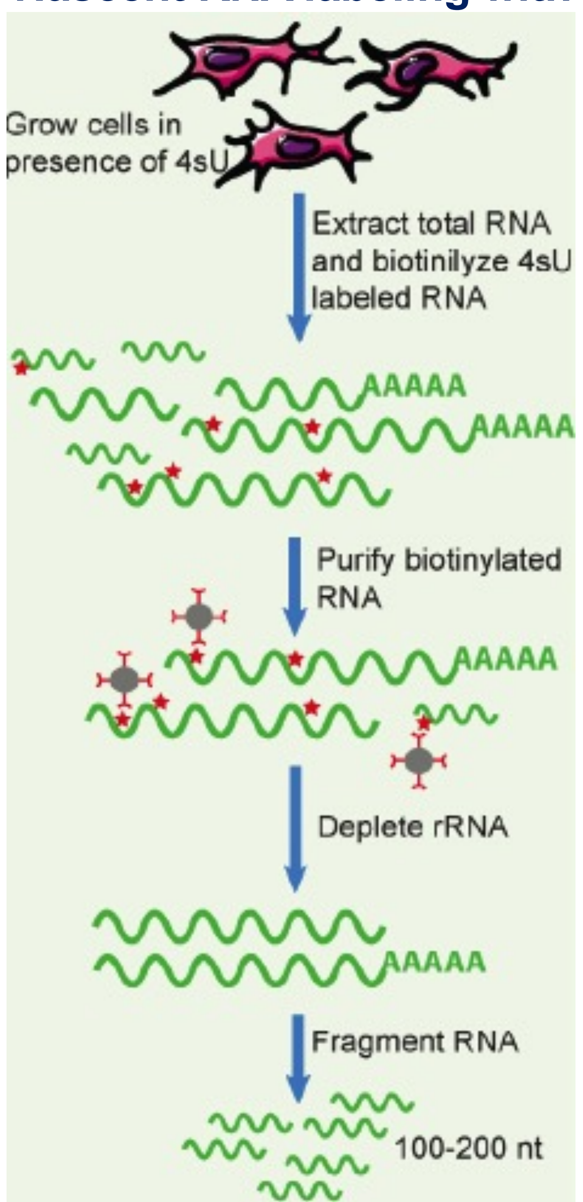
I. Isolation of PolII-bound RNAs



Churchman and Weissman, Nature, 2011

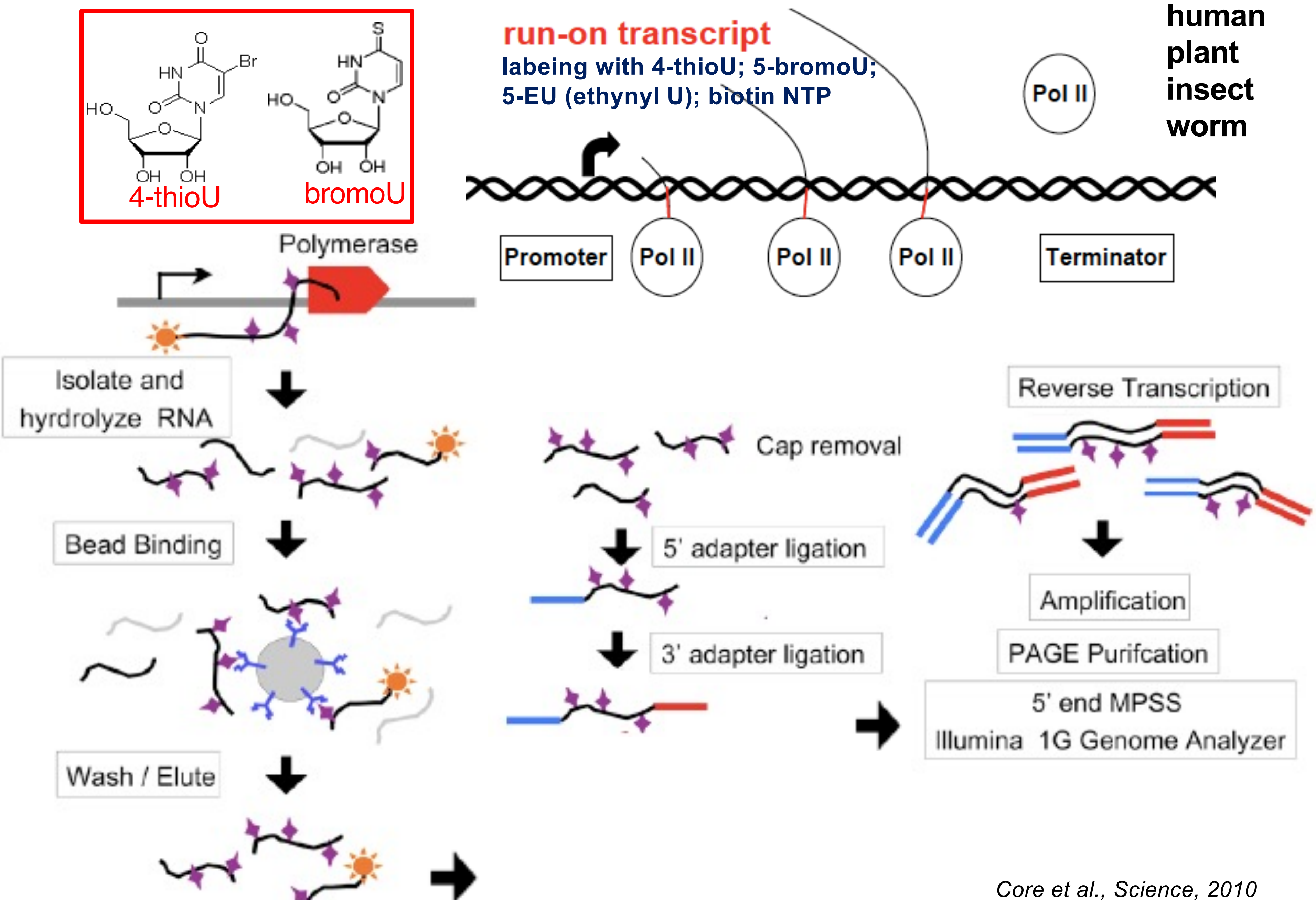
GRO-seq

II. Nascent RNA labeling with 4sU



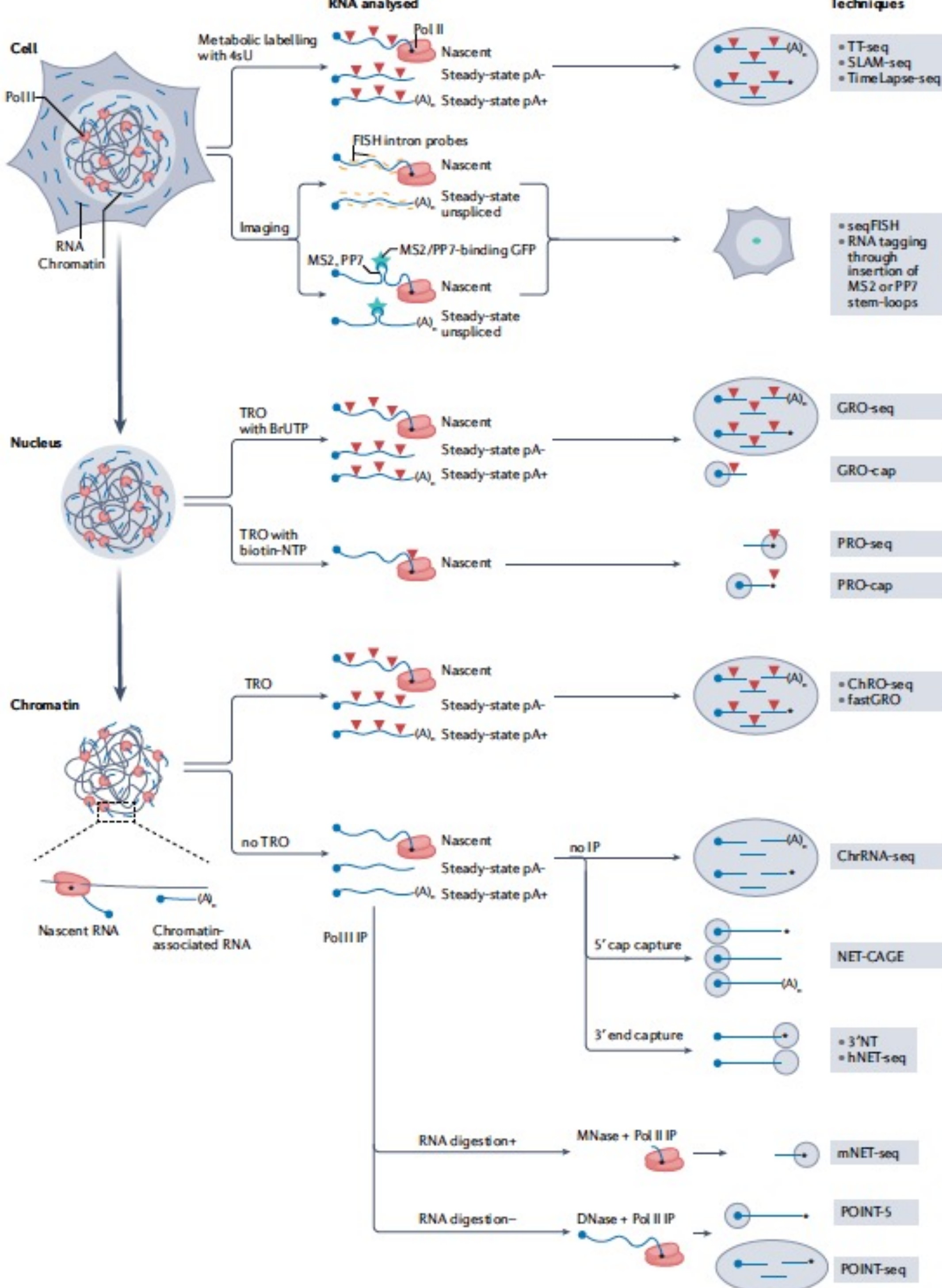
Spicuglia et al., Methods, 2013

Analysis of Nascent Transcripts: GRO-seq



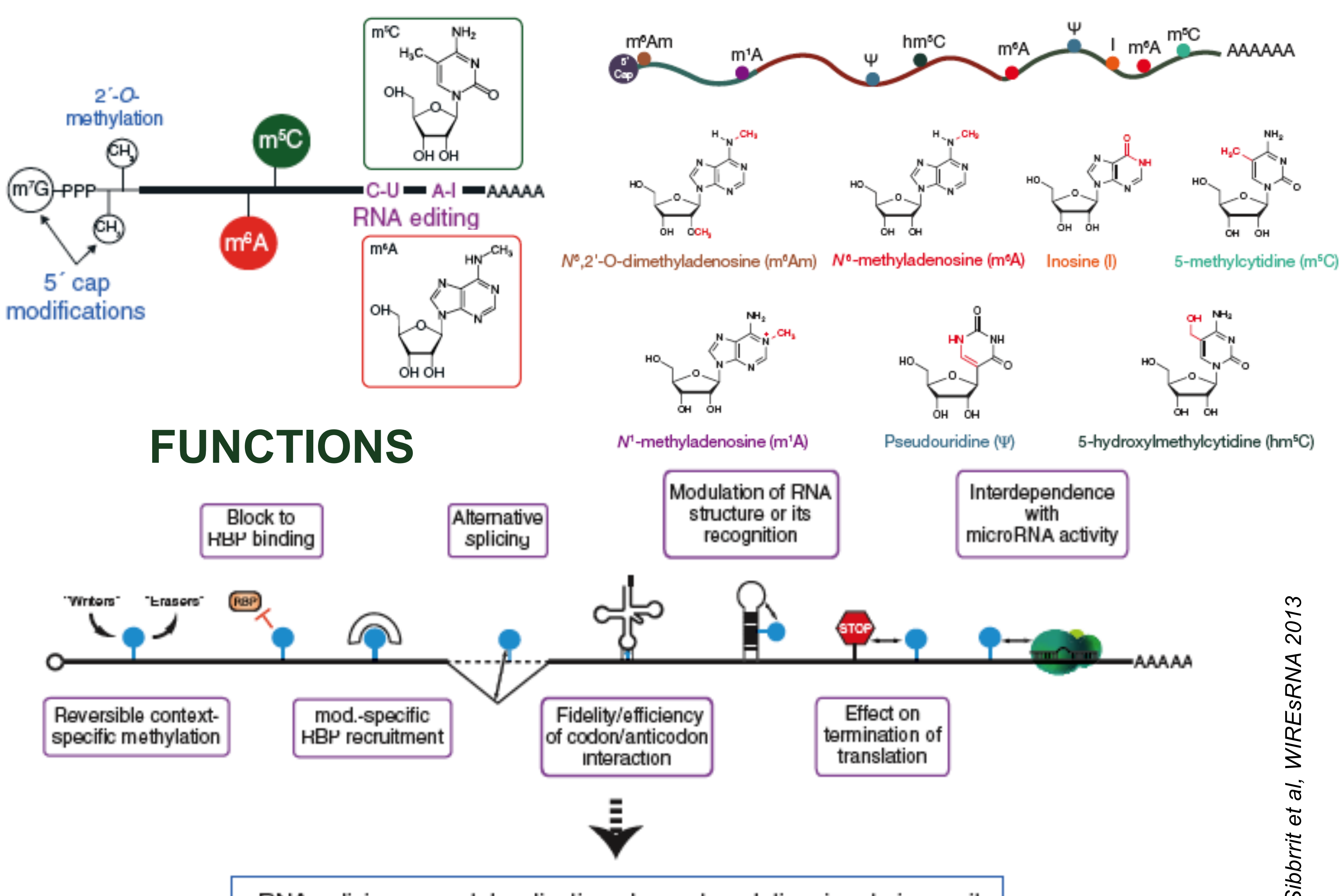
Core et al., Science, 2010

Nascent RNA analysis in mammalian cells

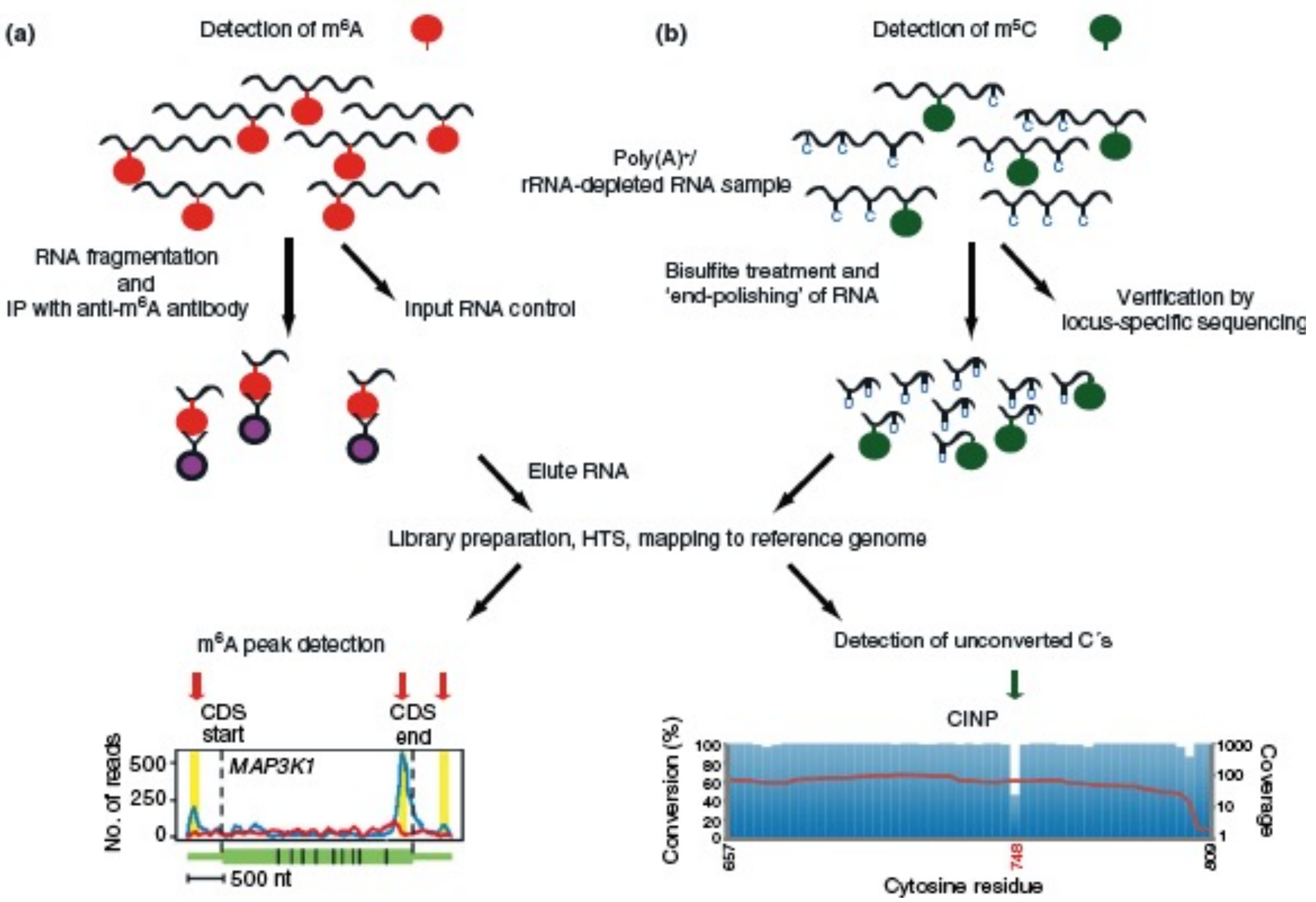


Nojima and Proudfoot, Nat Rev Mol Cell Biol, 2022

RNA modifications

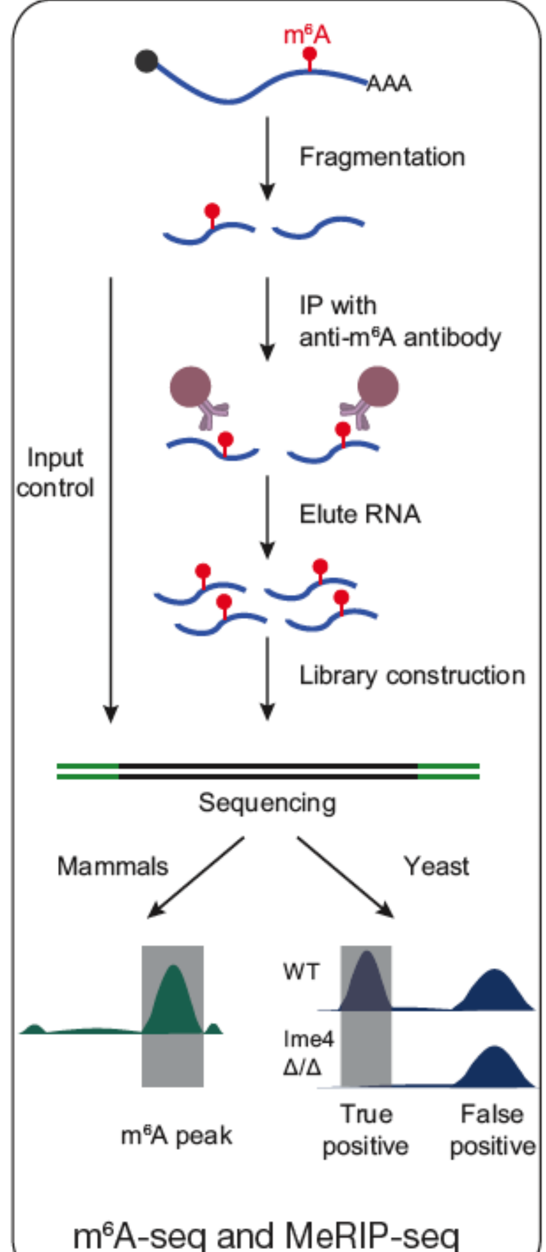


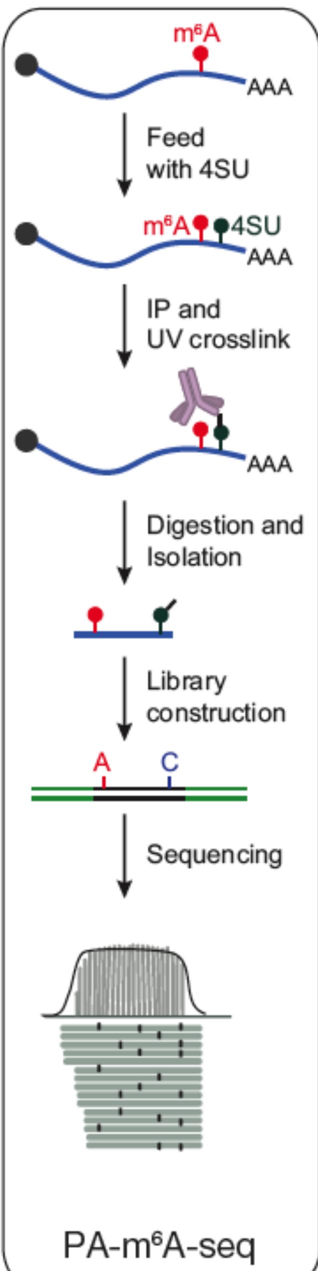
RNA modifications

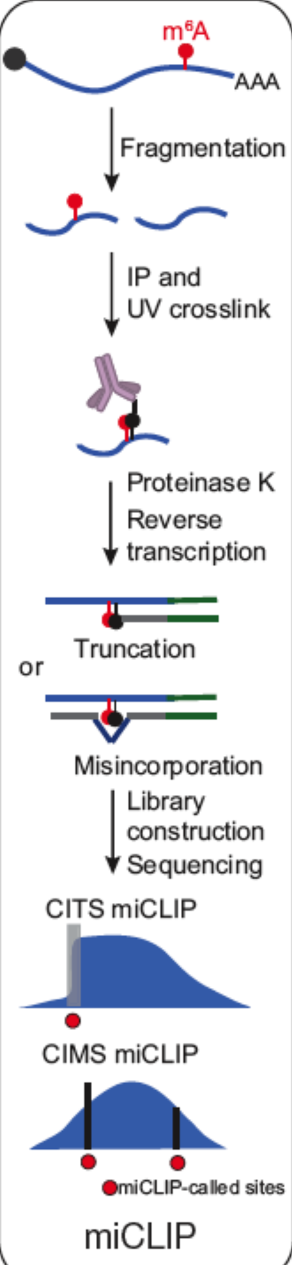


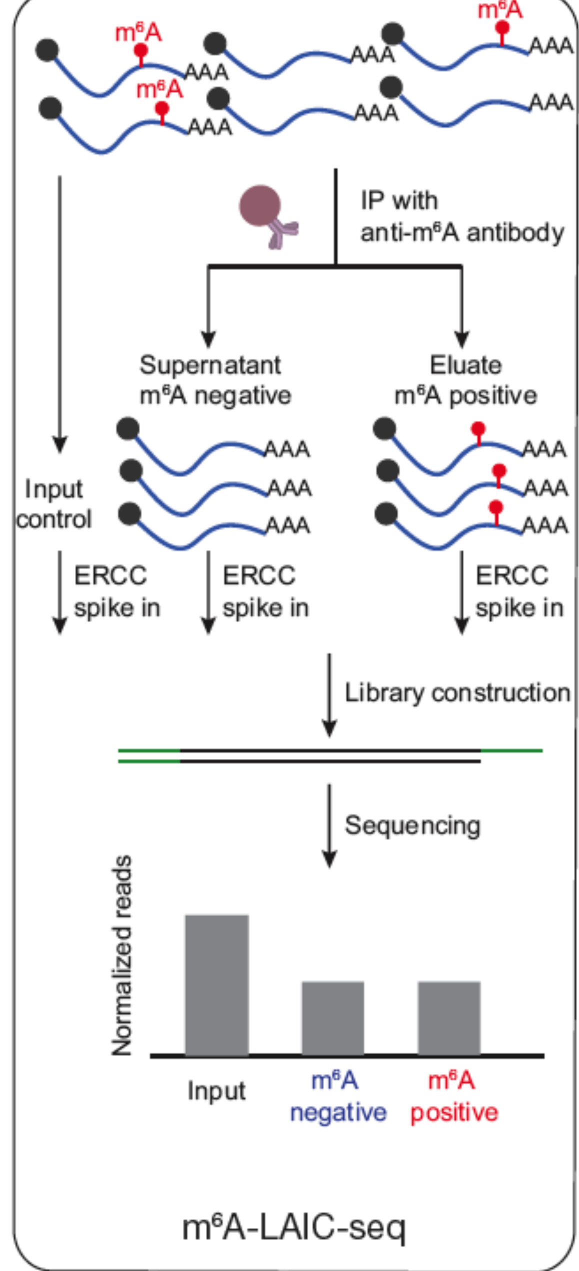
Sibbrrit et al, WIREsRNA 2013

m^6A RNA-seq









Li at al, Nat Methods, 2017

Antibody-free m6A-seq

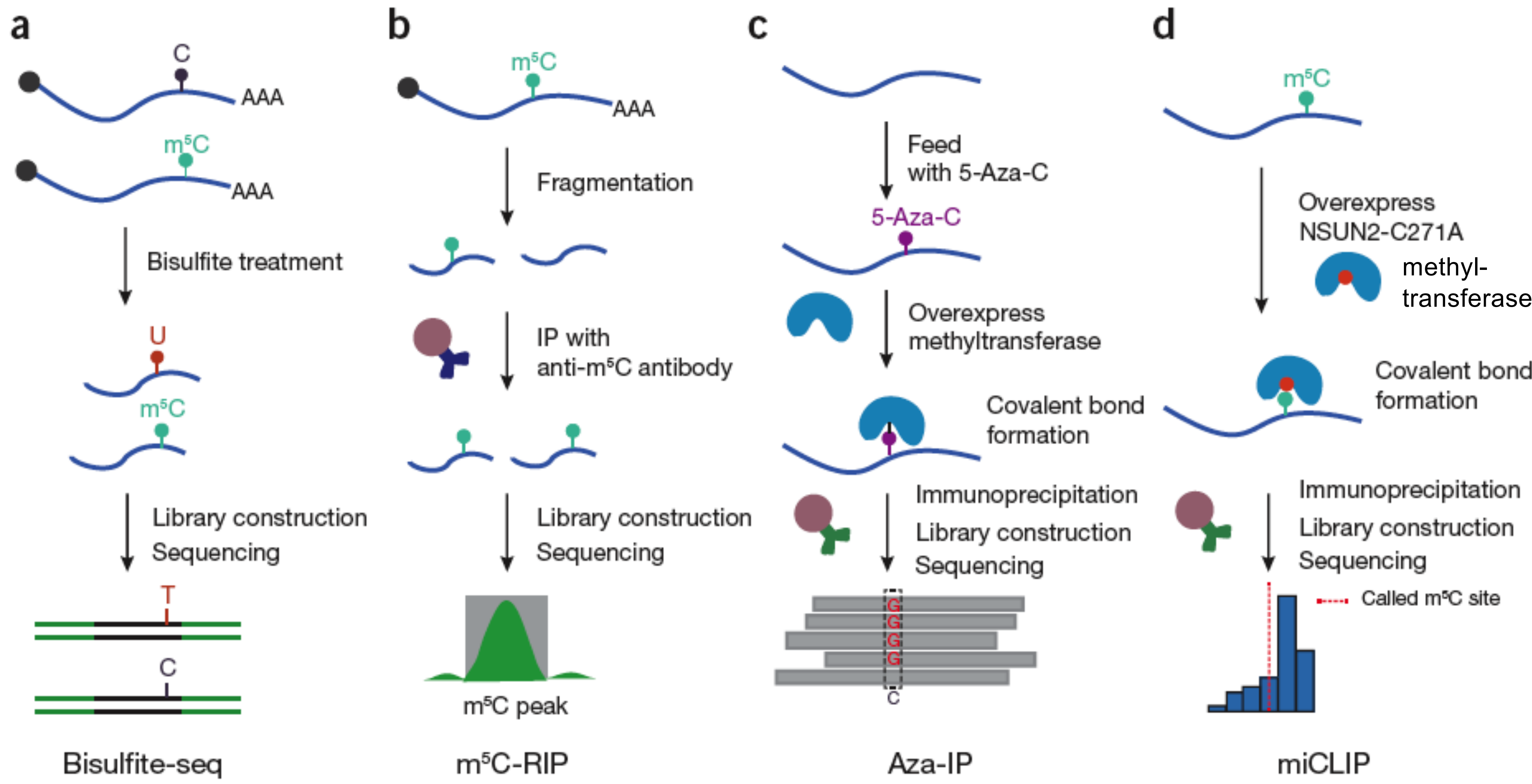
DART-seq

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

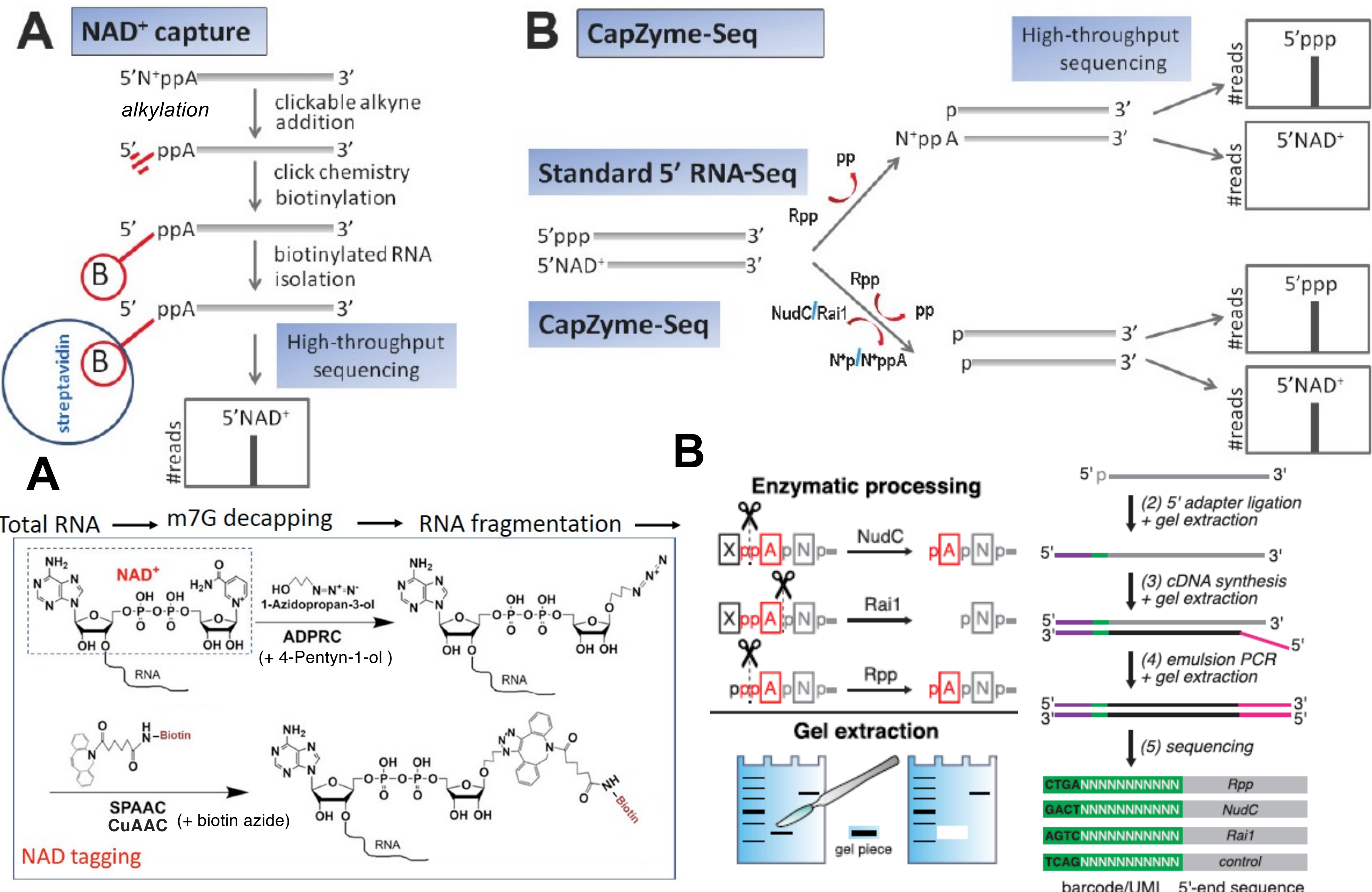
Meyer, Nature Methods 2019

m^5C 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:

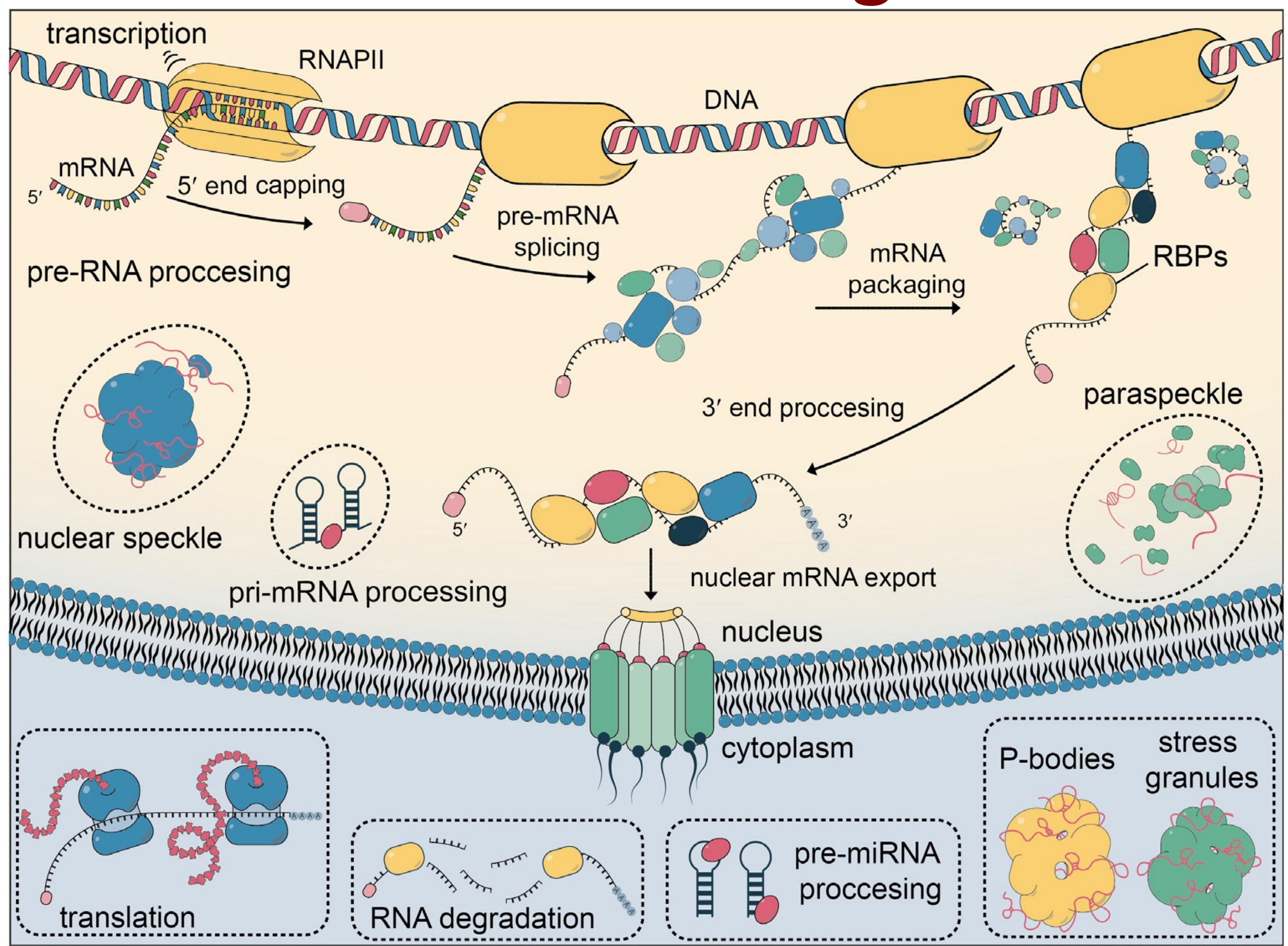
RNA-proteins

RNA-DNA

RNA-RNA

RNA structure

RBP - RNA Binding Proteins

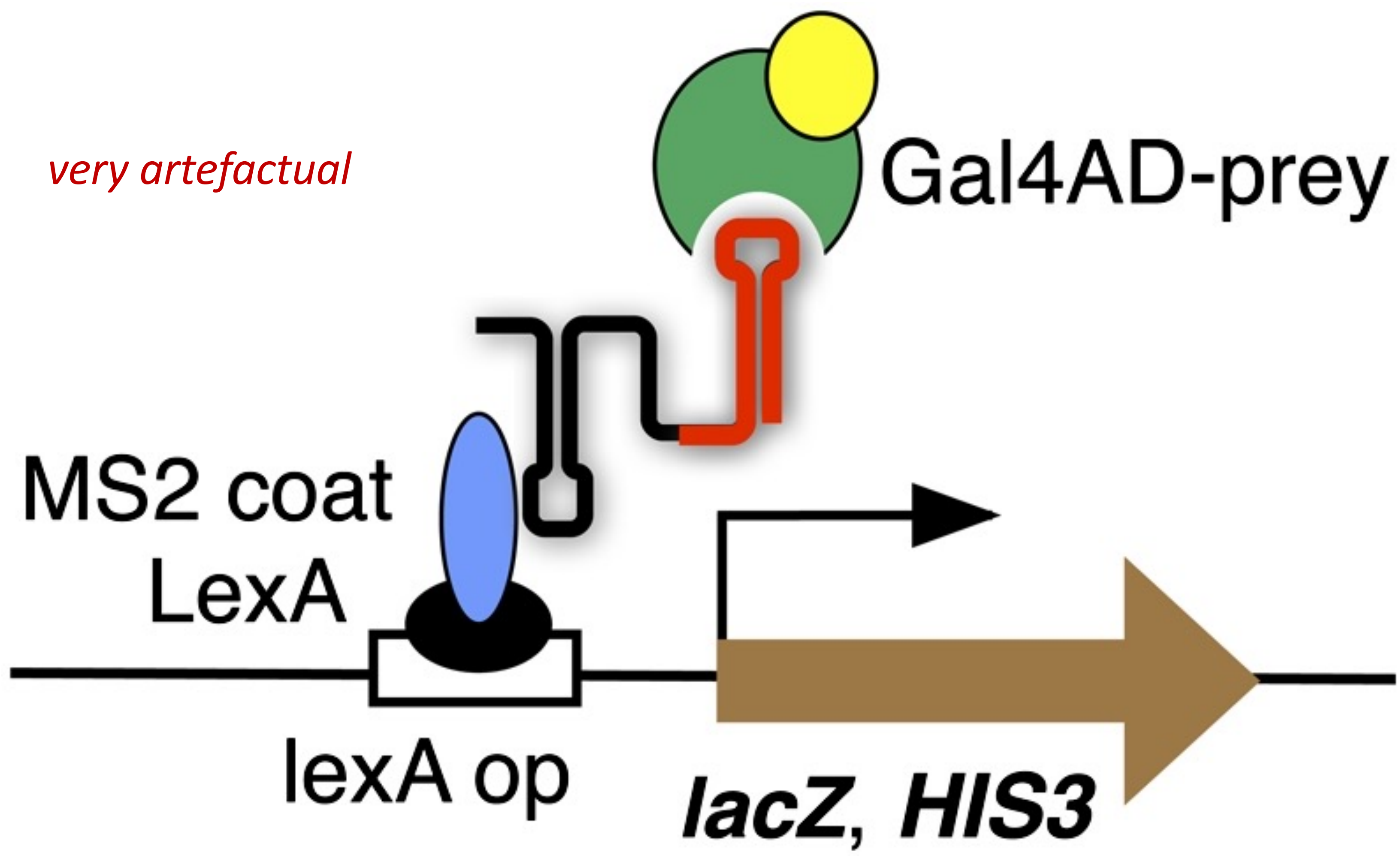


Kilchert et al., WiRES RNA, 2020

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

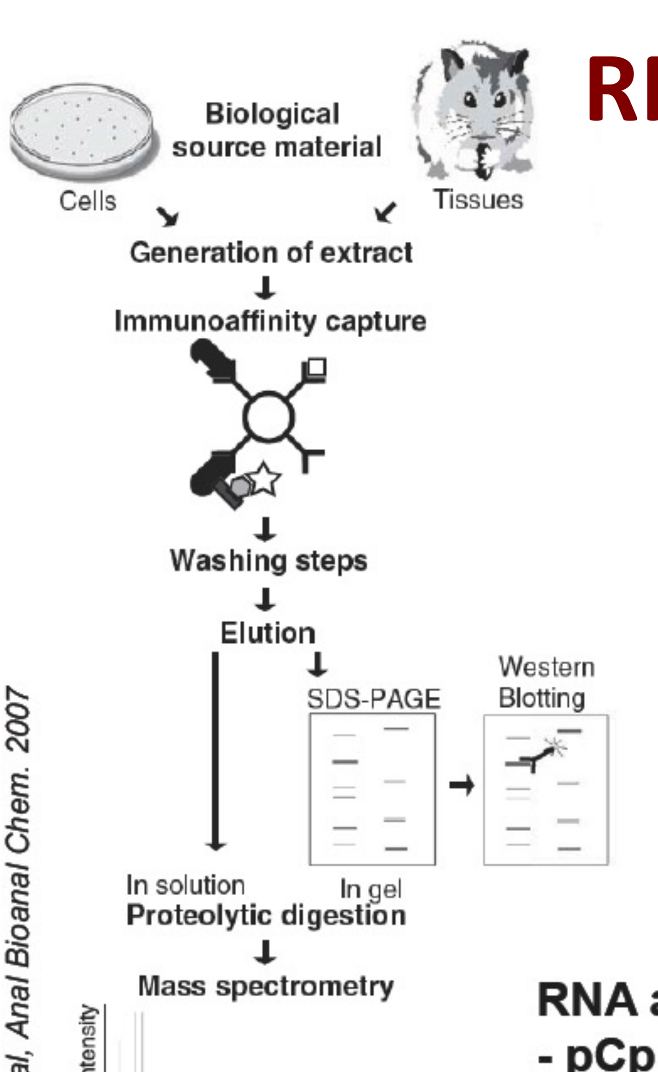
Genetic Screen- Yeast Three Hybrid

very artefactual



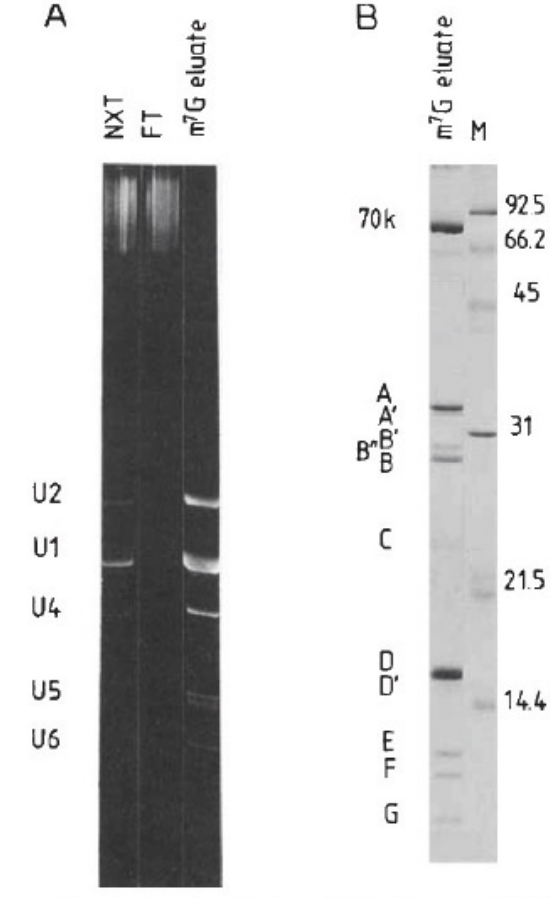
RNA insert is expressed in the context of **RNA** vector sequences tethered upstream of ***lacZ*** and ***HIS3*** reporter genes via a **MS2** coat–**LexA** fusion protein. Gene activation depends on binding of the **Gal4** activation domain–**prey** fusion protein.

RNP Immunoprecipitation (IP)



With specific antibodies or using tagged proteins

U snRNPs with anti-TMG cap antibody



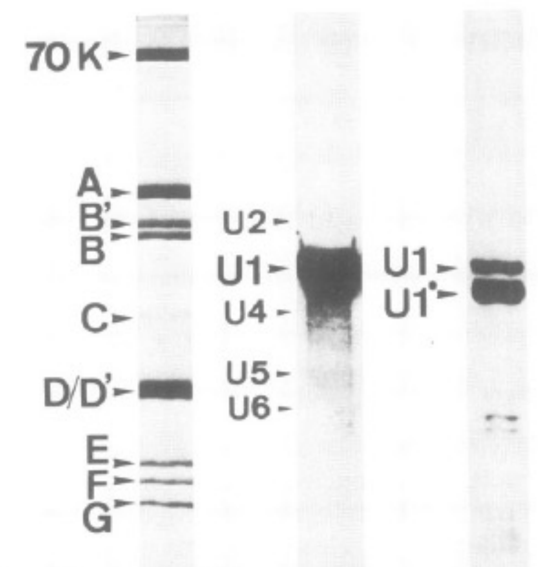
Bochnig et al, Eur. J Biochem. 1987
(Luhrmann's lab)

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

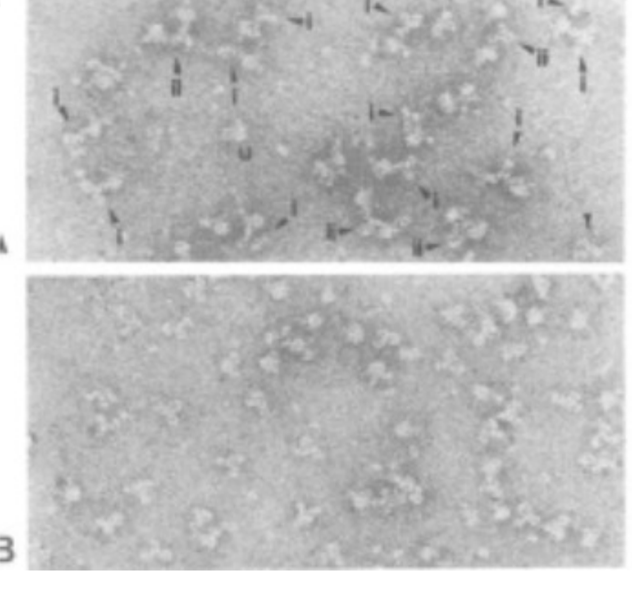
IP of U1 snRNP with α-70K

(U1 RNP specific protein)

Immunoaffinity +ion exchange



Electron Microscopy



IP of snRNPs with α-TMG cap

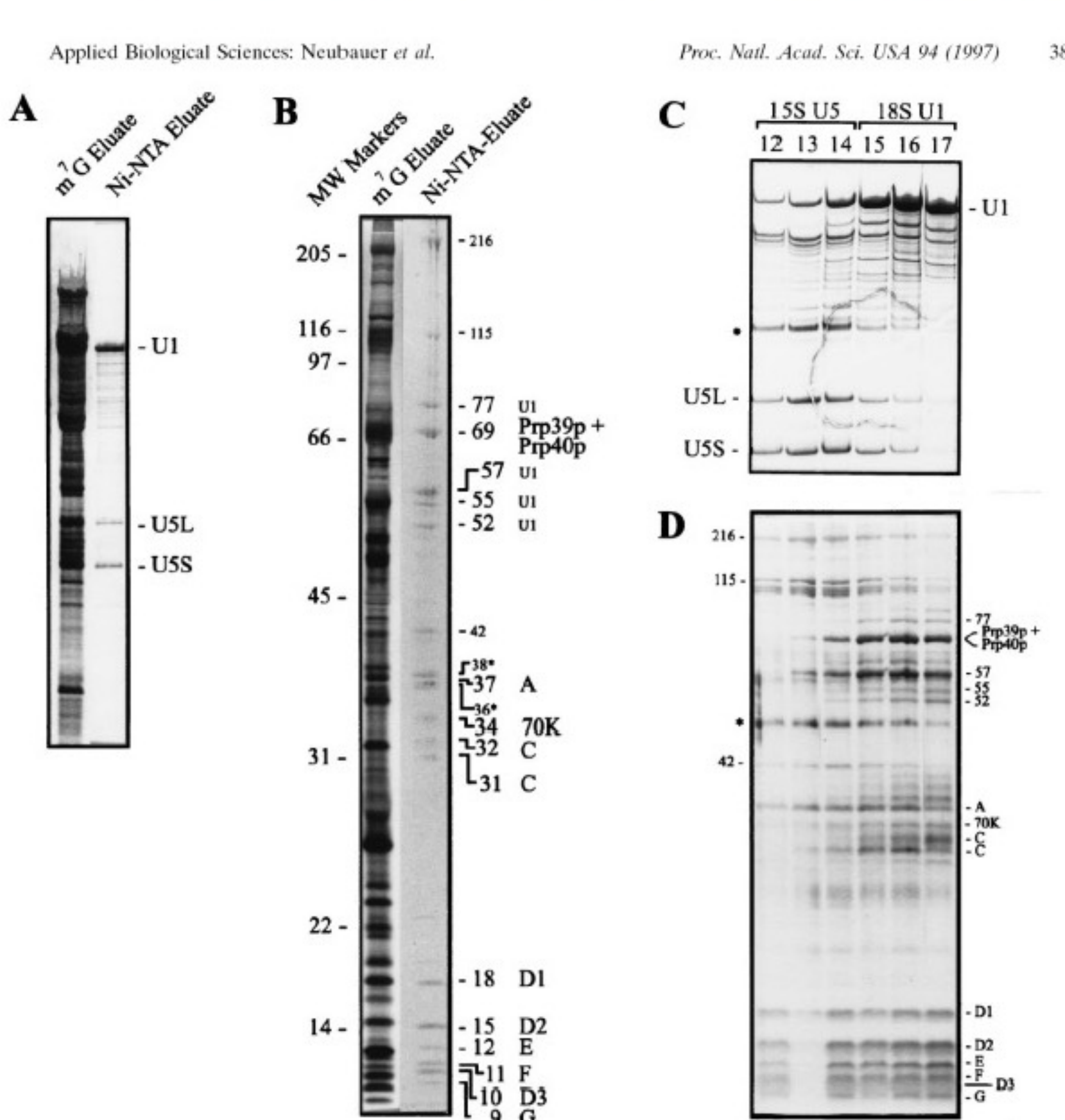
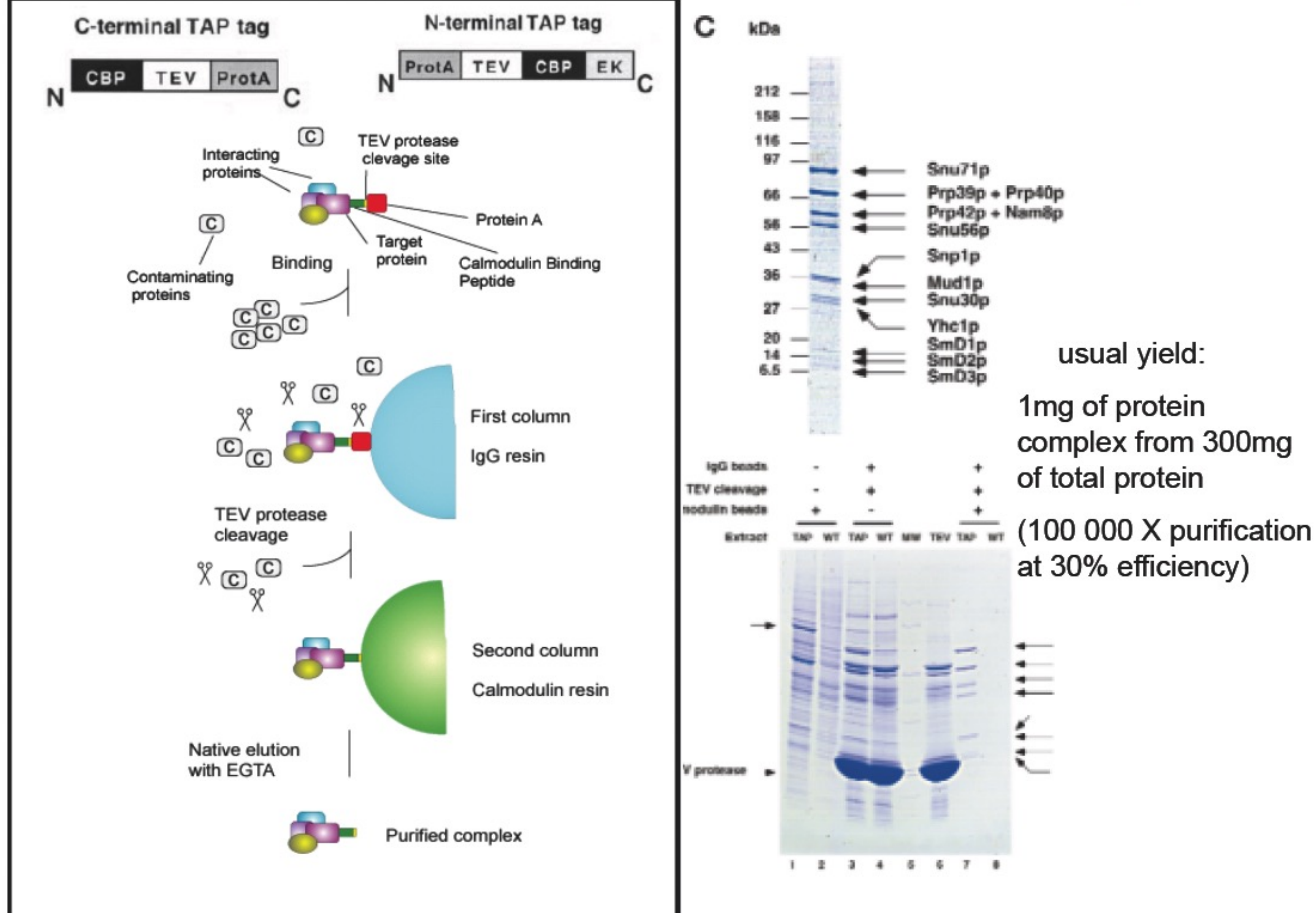
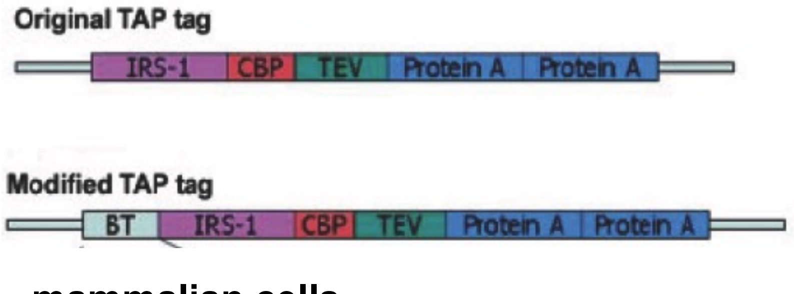


FIG. 1. Purification of U1 snRNPs from *S. cerevisiae*. (*A*) Silver staining of snRNAs eluted from anti-m$_3$G-cap (m^{7}G eluate) and Ni-NTA affinity

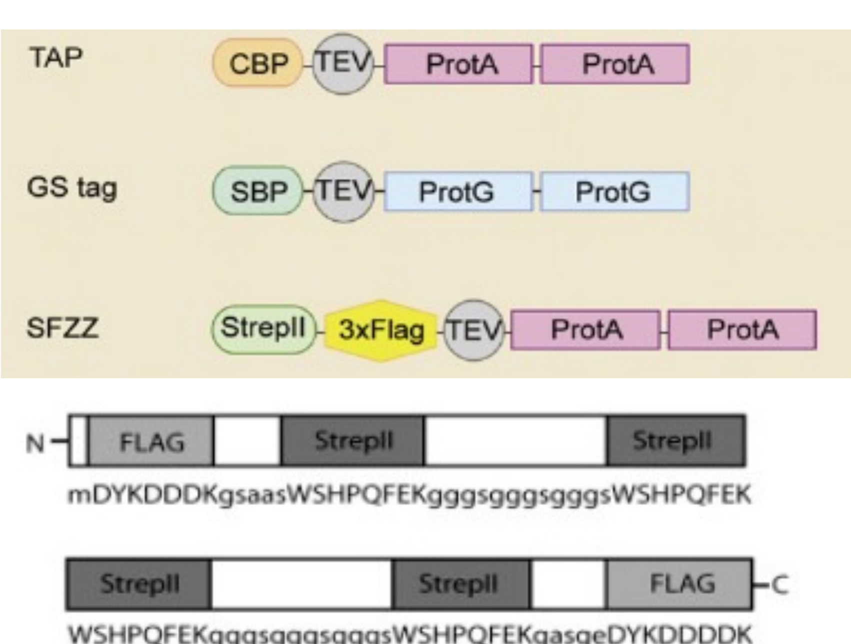
Tandem Affinity Purification (TAP)



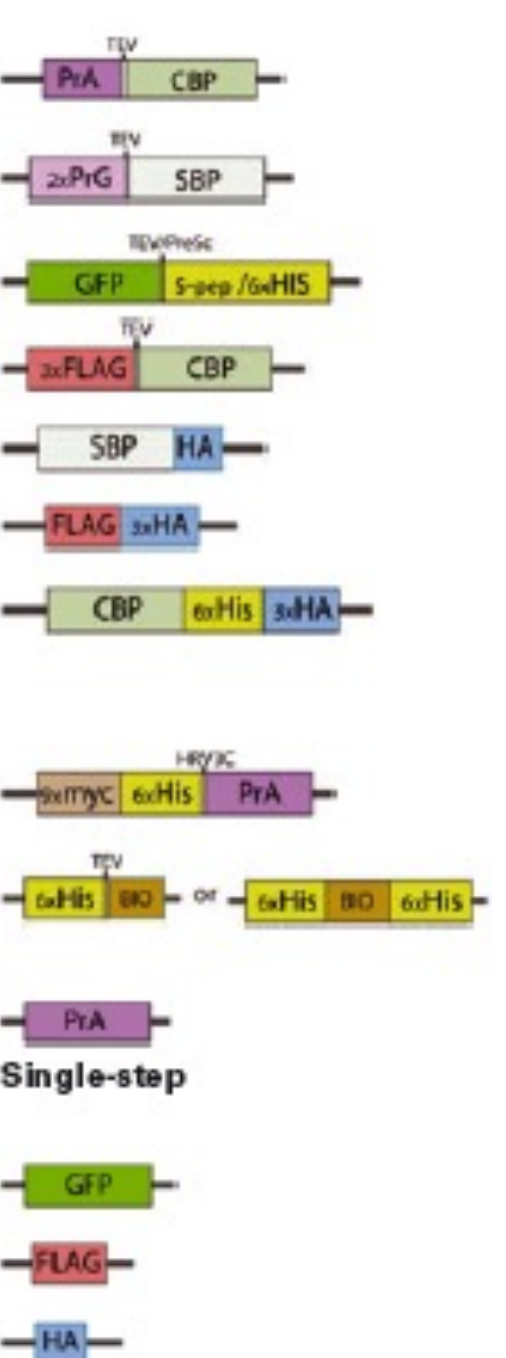
Modified TAP tags



mammalian cells

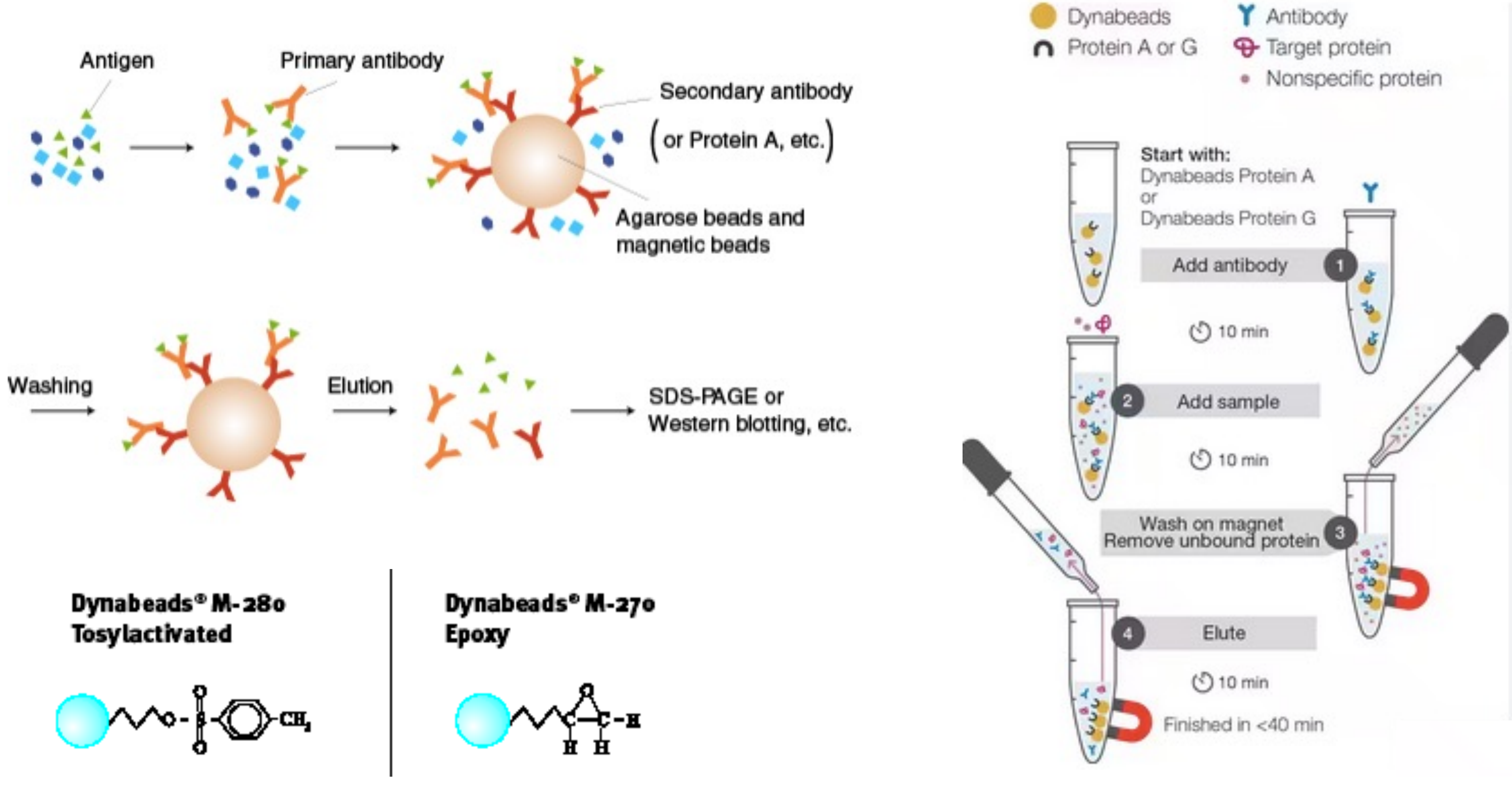


Drakas et al., Proteomics, 2005
Van Leene et al., TiPISci, 2008;
Gloeckner et al, Proteomics, 2007
Oeffinger, Proteomics, 2012



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

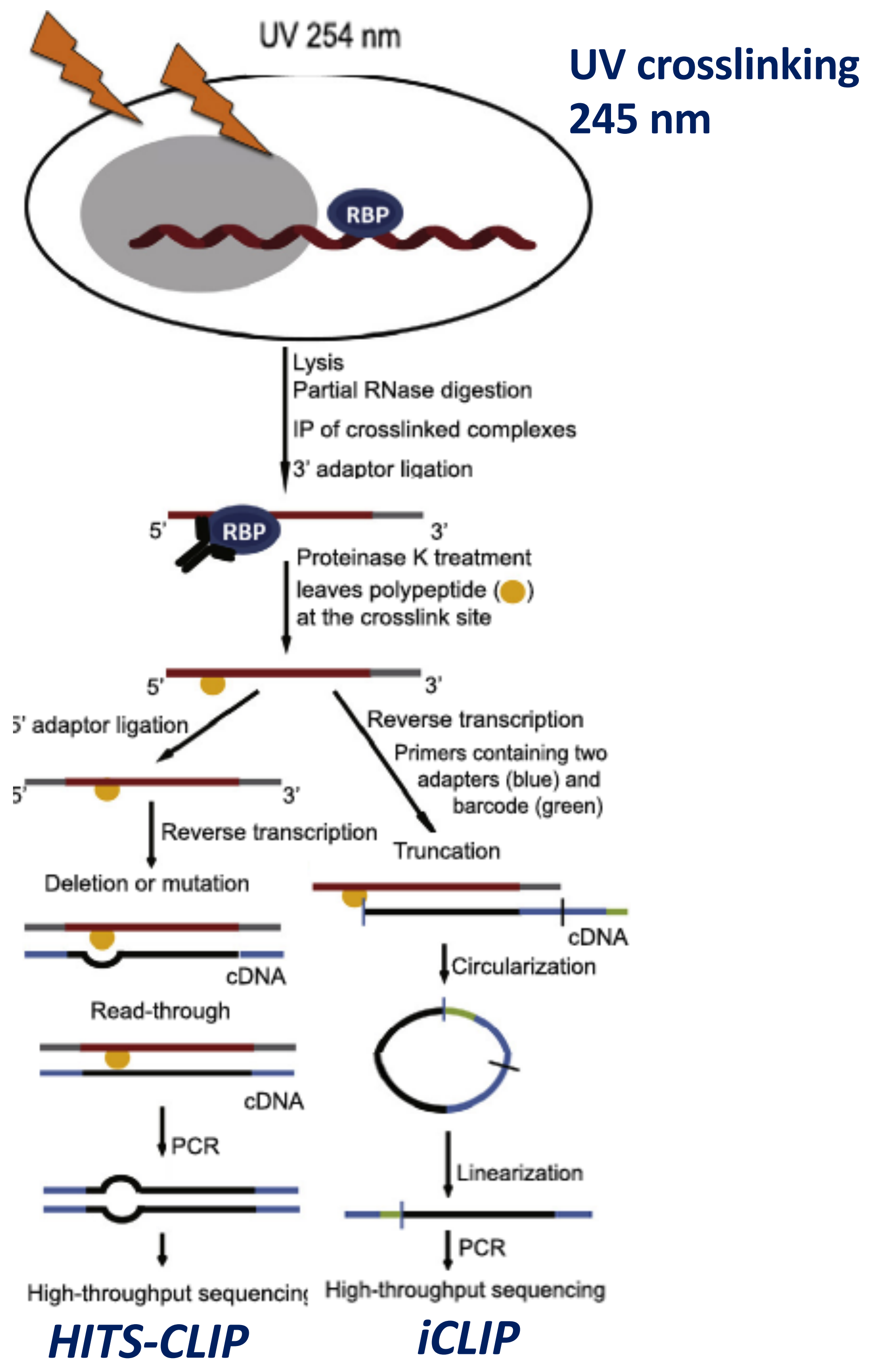
CrossLinking and ImmunoPrecipitation

HITS-CLIP

High-Throughput Seq CLIP

iCLIP

individual nucleoside resolution CLIP

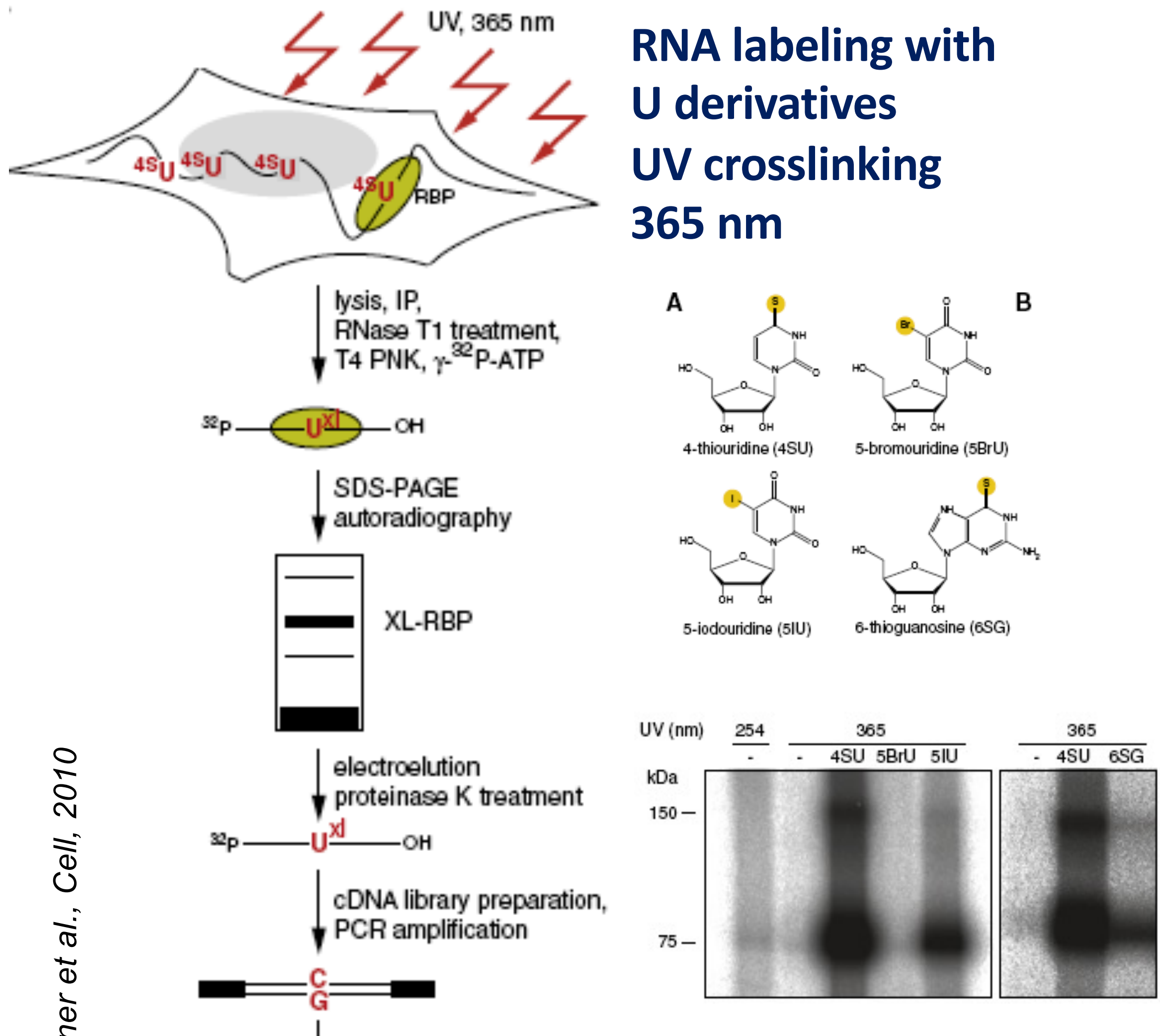


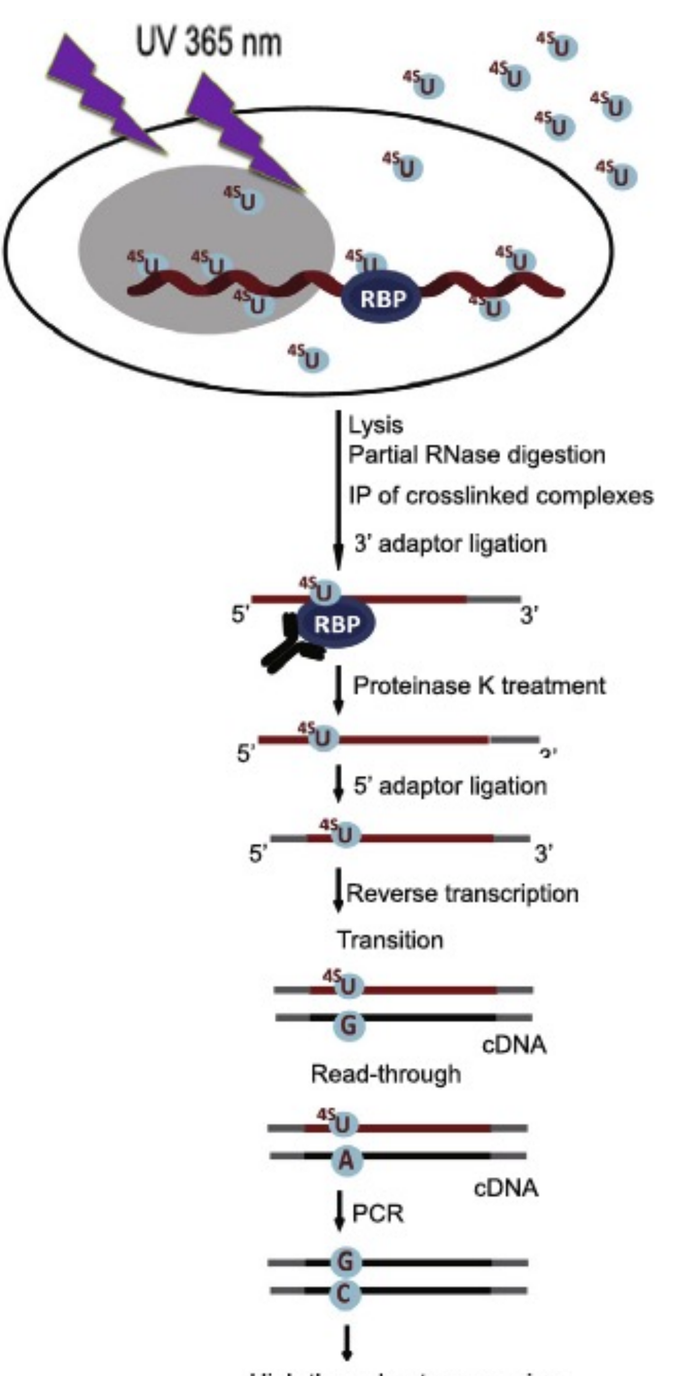
Li et al., Genome Proteome Bioinformatics, 2014

PAR-CLIP

PhotoActivatable ribonucleoside–enhanced CLIP

RNA labeling with U derivatives
UV crosslinking 365 nm

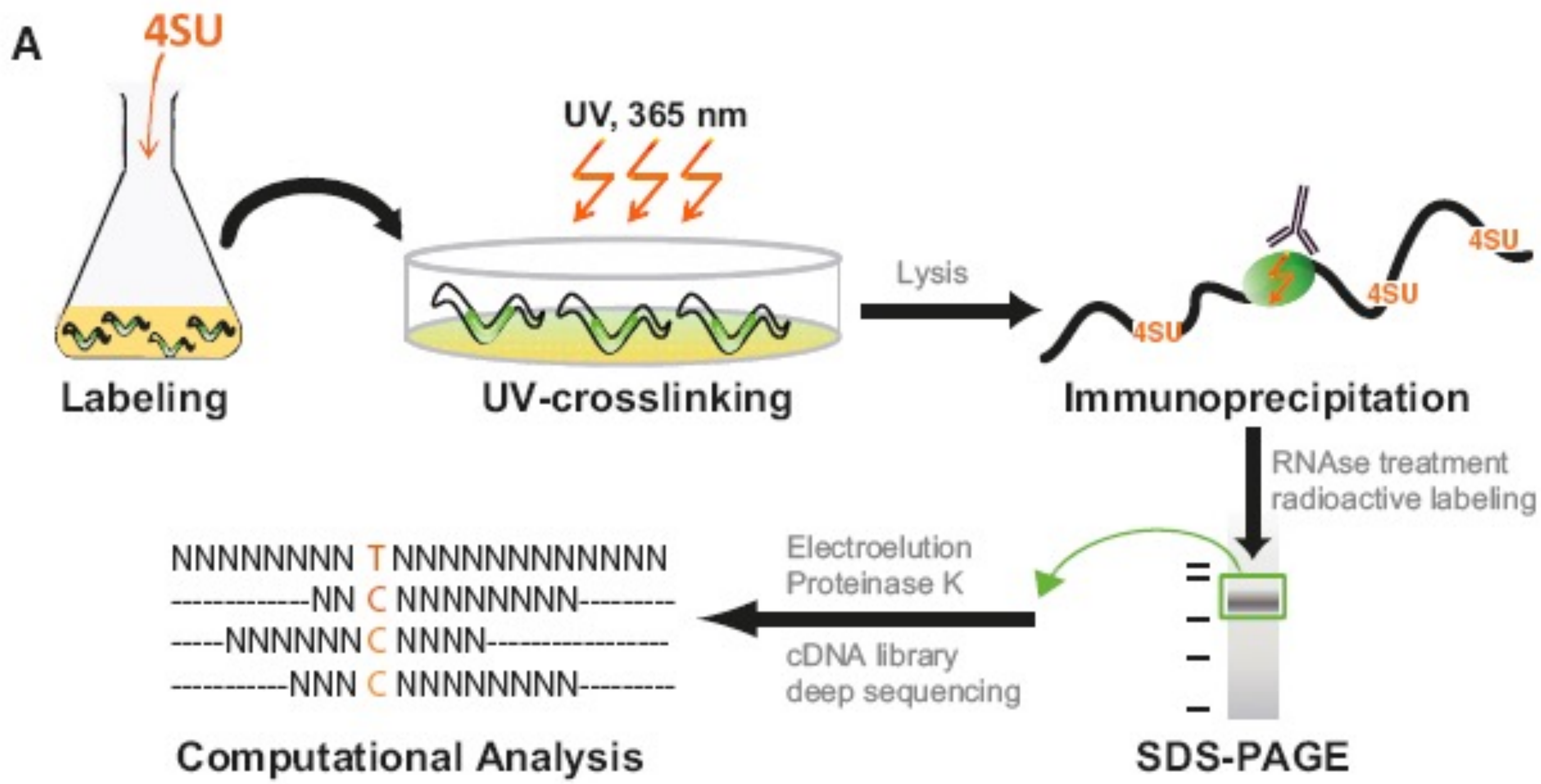




Hafner et al., Cell, 2010

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

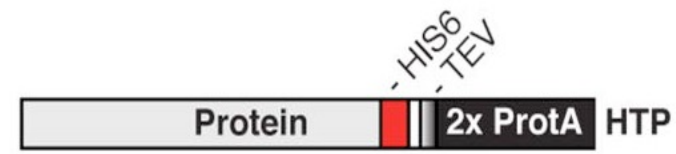
in vivo PAR-CLIP

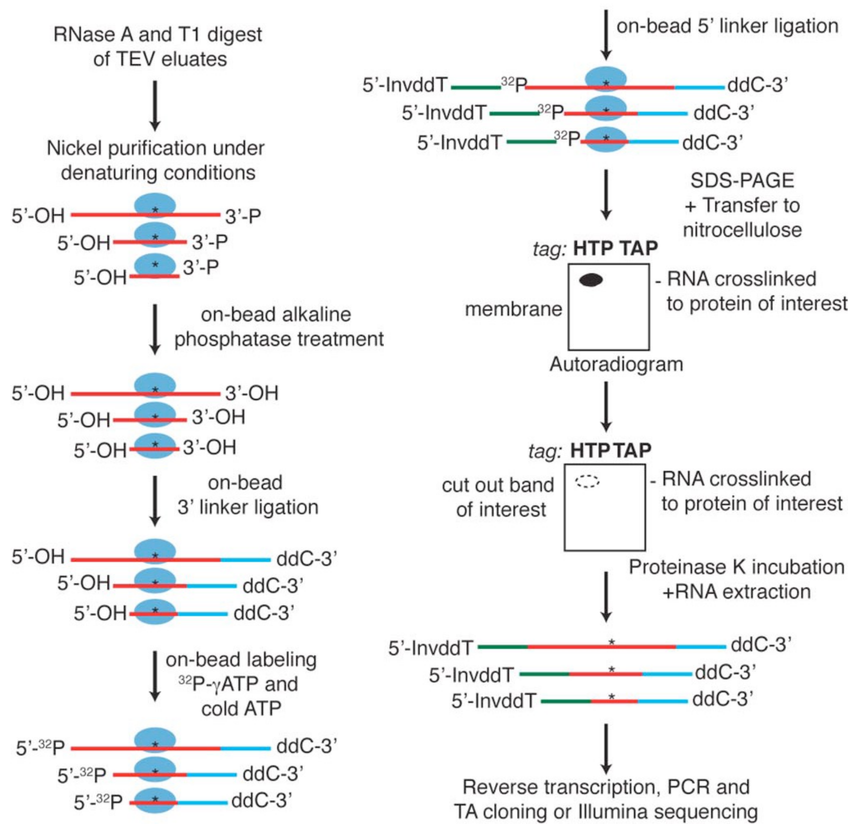


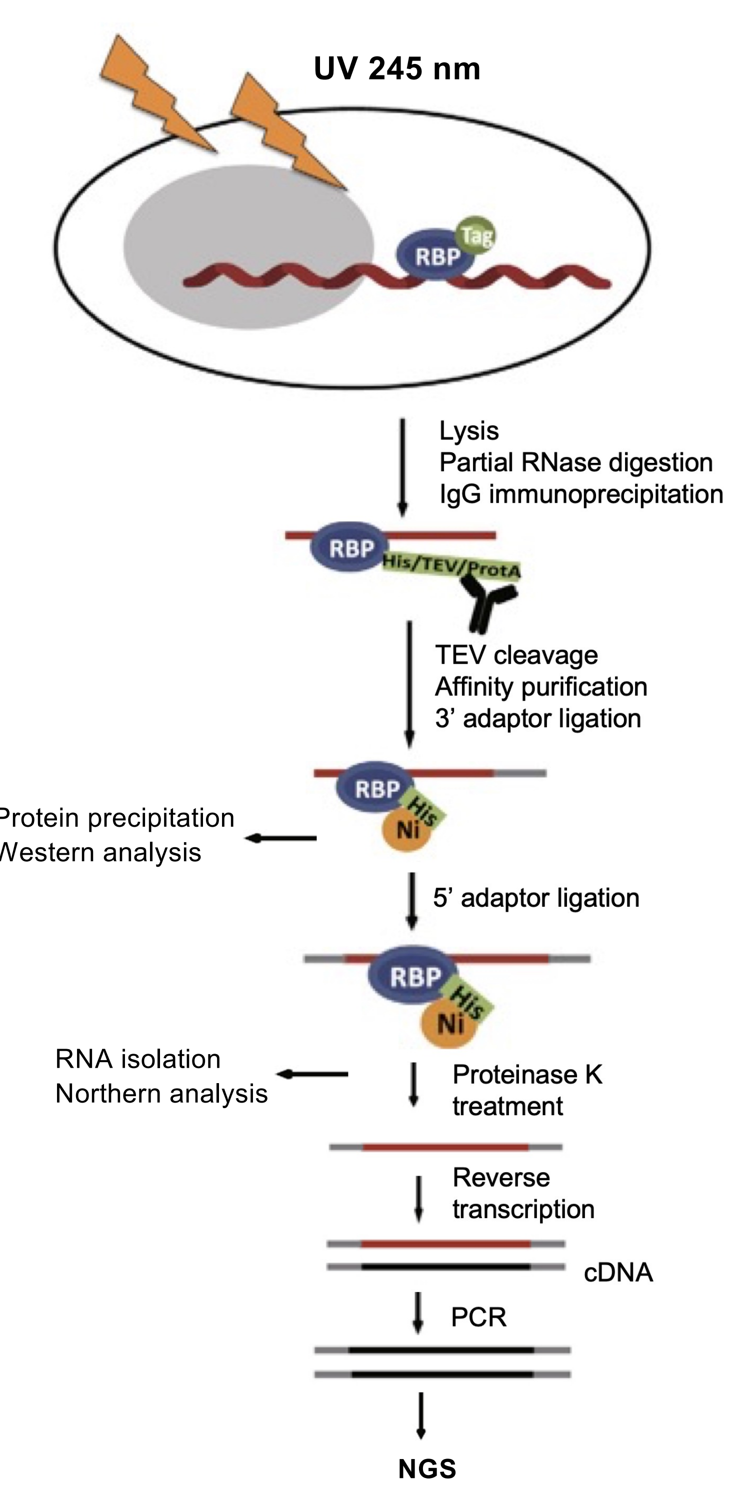
Jungkamp et al., Mol Cell, 2011

CRAC

CRosslinking and Analysis of cDNA

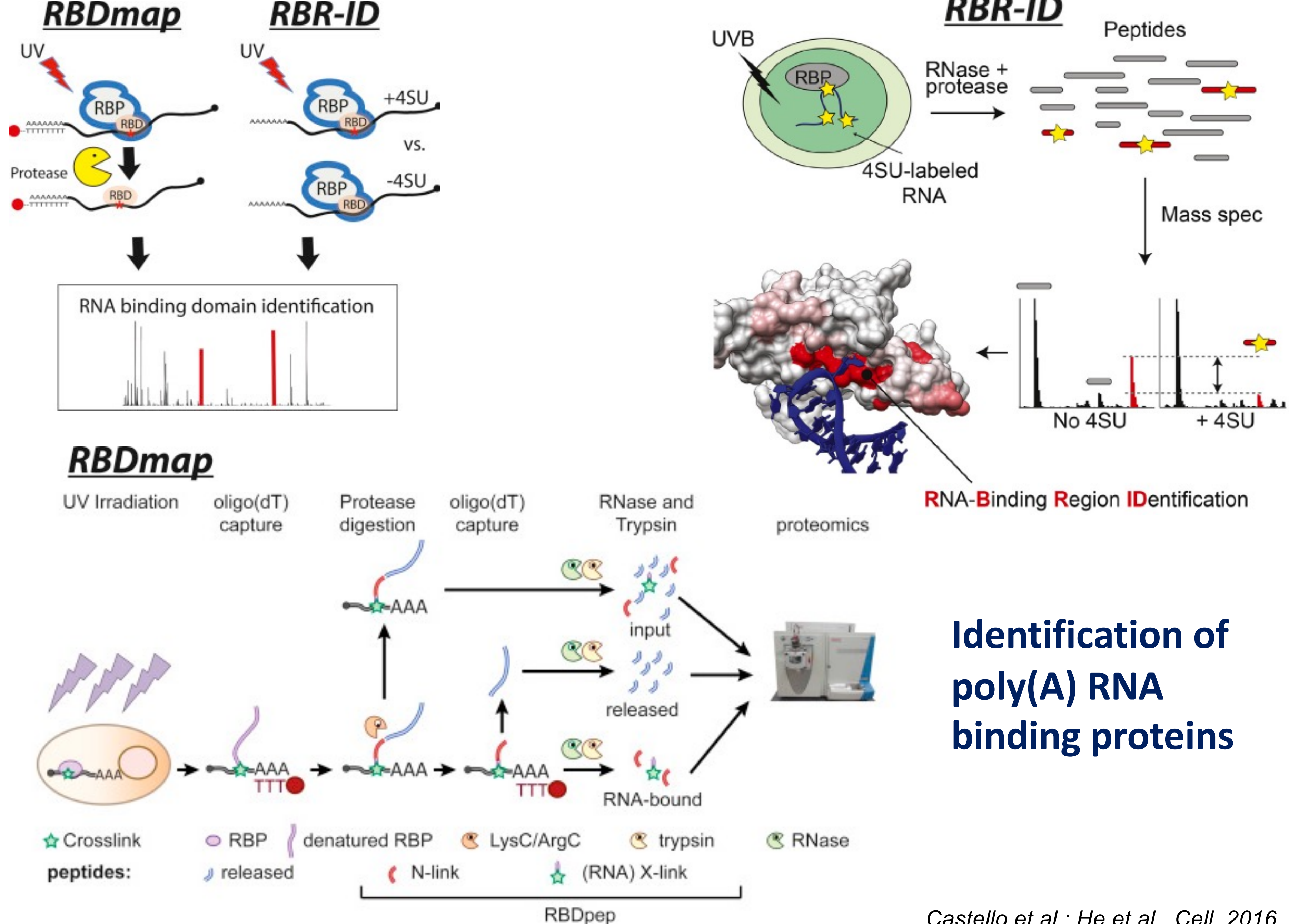






Granneman et al., PNAS, 2009
Li et al., Genome Proteome Bioinformatics, 2014

mRNA binding proteome, mRPBome

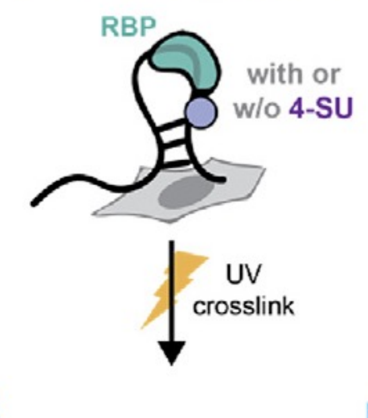


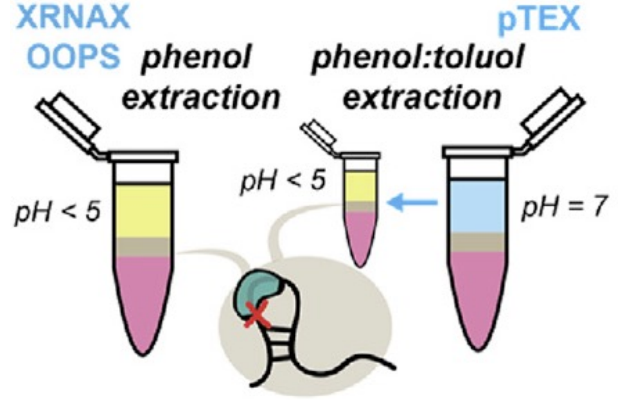
Identification of poly(A) RNA binding proteins

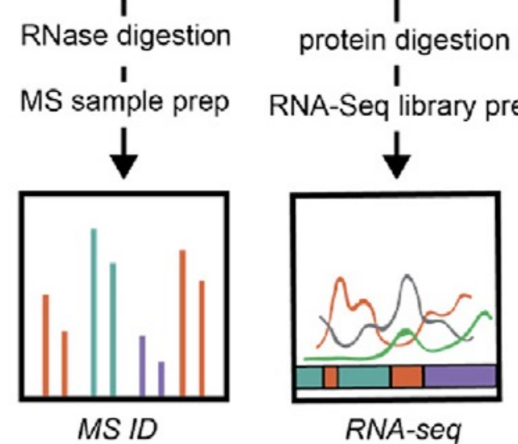
Castello et al.; He et al., Cell, 2016

OOPS, XRNAX, pTEX

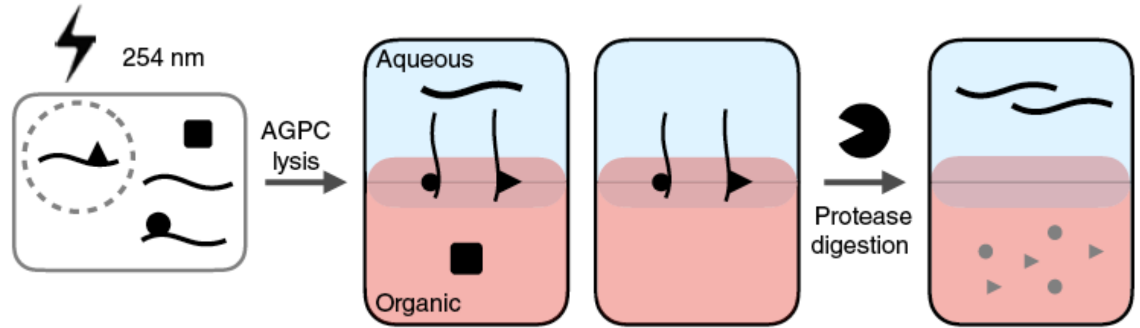
RNP interactome, RPBome



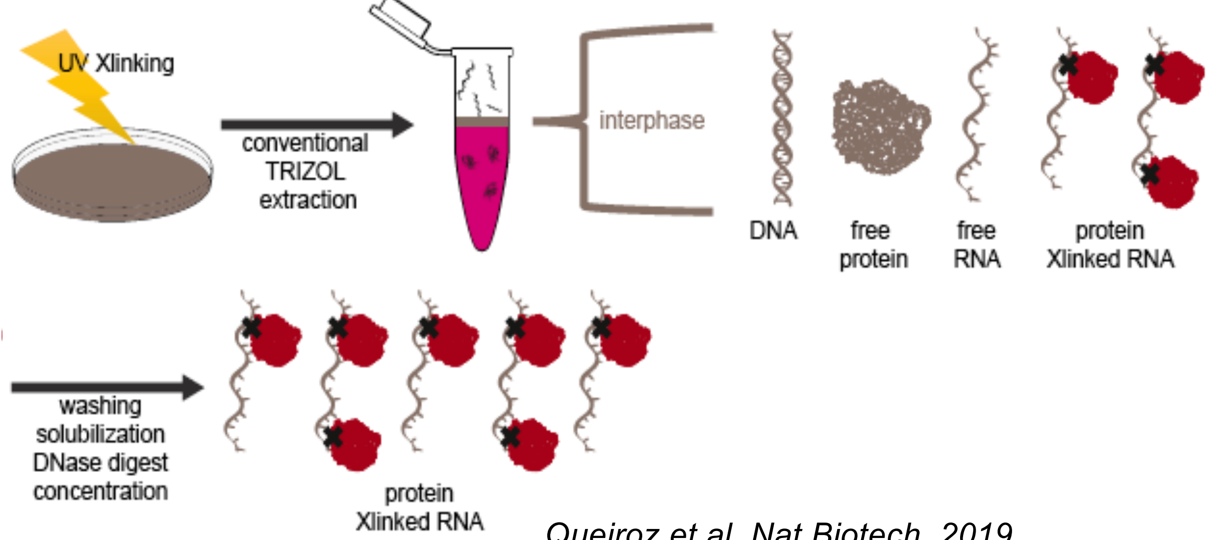




OOPS - orthogonal organic phase separation

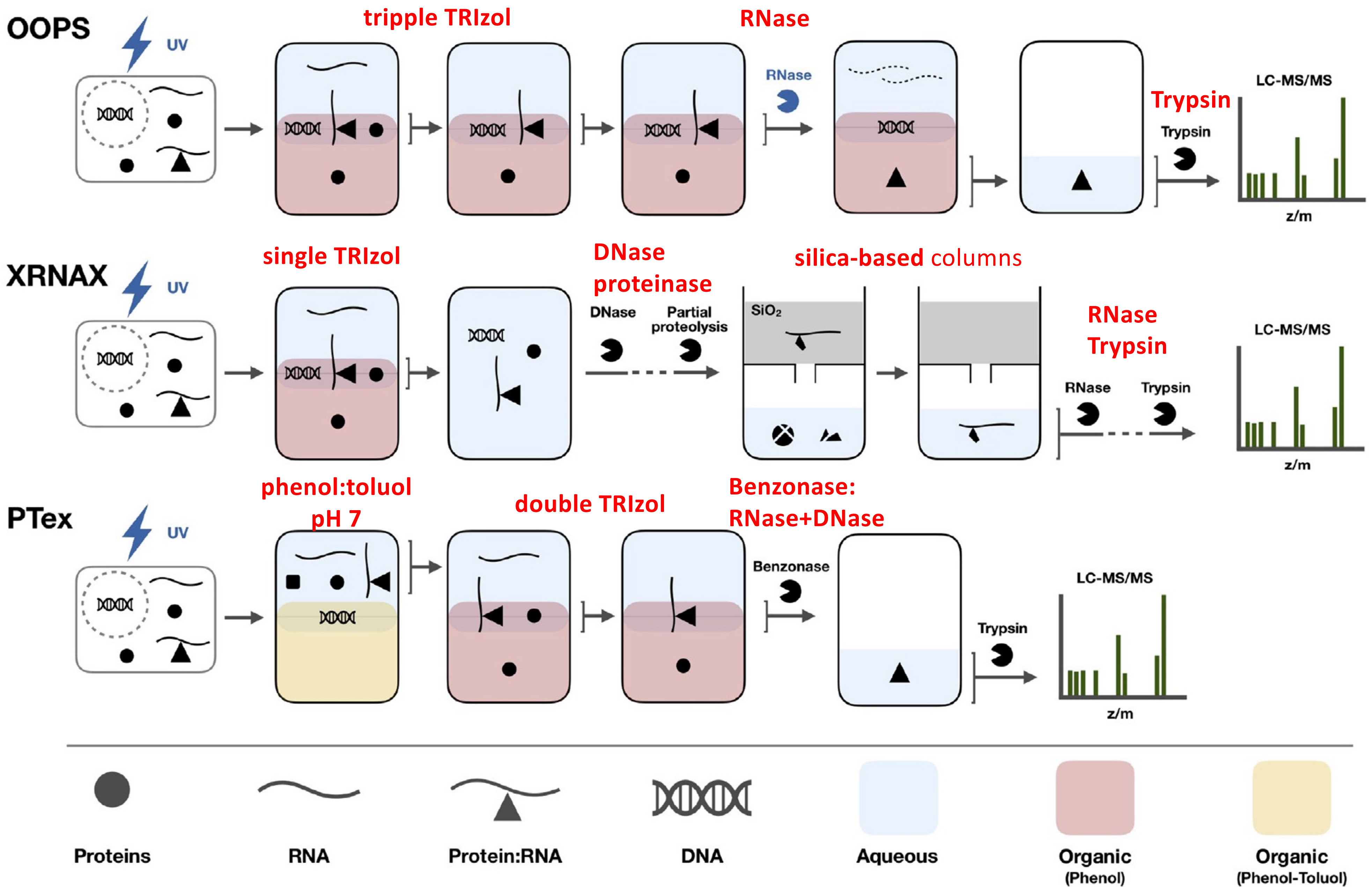


XRNAX - Cross-Linked RNA eXtraction



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

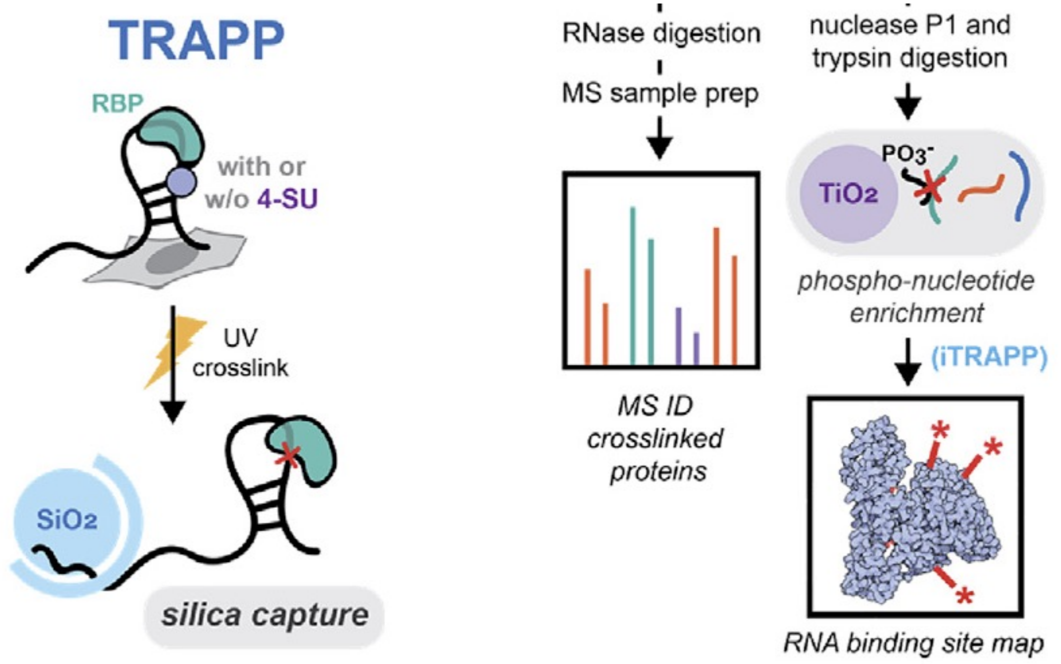
OOPS, XRNAX, PTex – organic phase separation



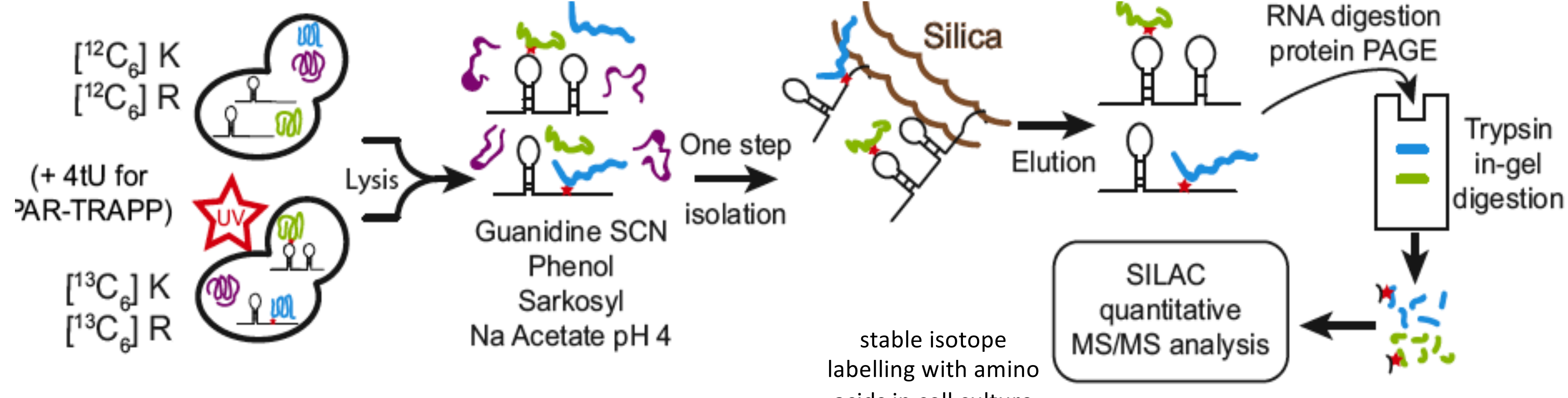
Smith et al. Curr Op Chem Biol, 2020

TRAPP, RNP interactome, RPBome

TRAPP/PAR-TRAPP - RNA-associated protein purification

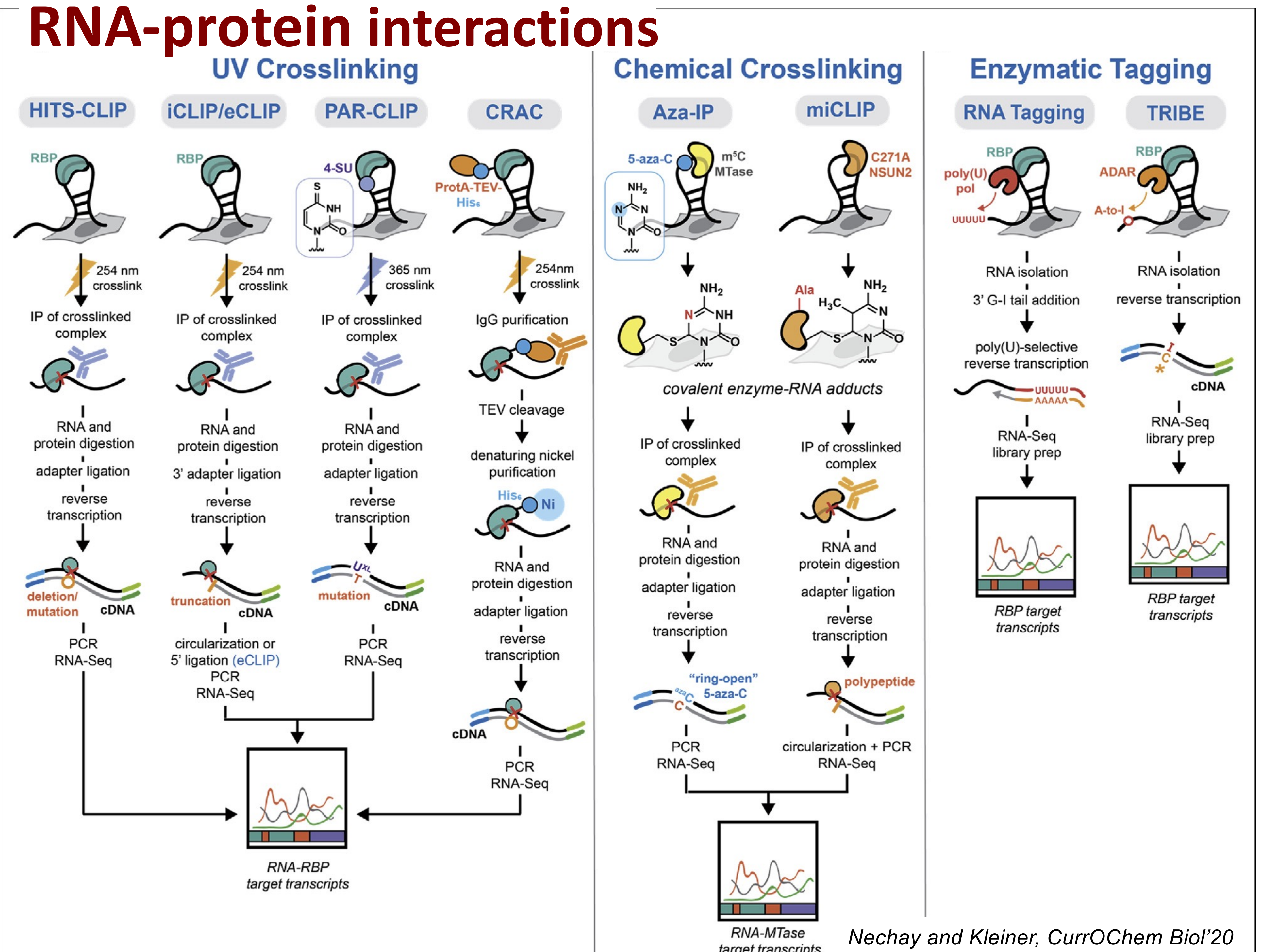


Queiroz et al, Nat Biotech, 2019
Shchepachev et al, Mol Sys Biol, 2019
Nechay and Kleiner, Curr Op Chem Biol, 2020



stable isotope labelling with amino acids in cell culture

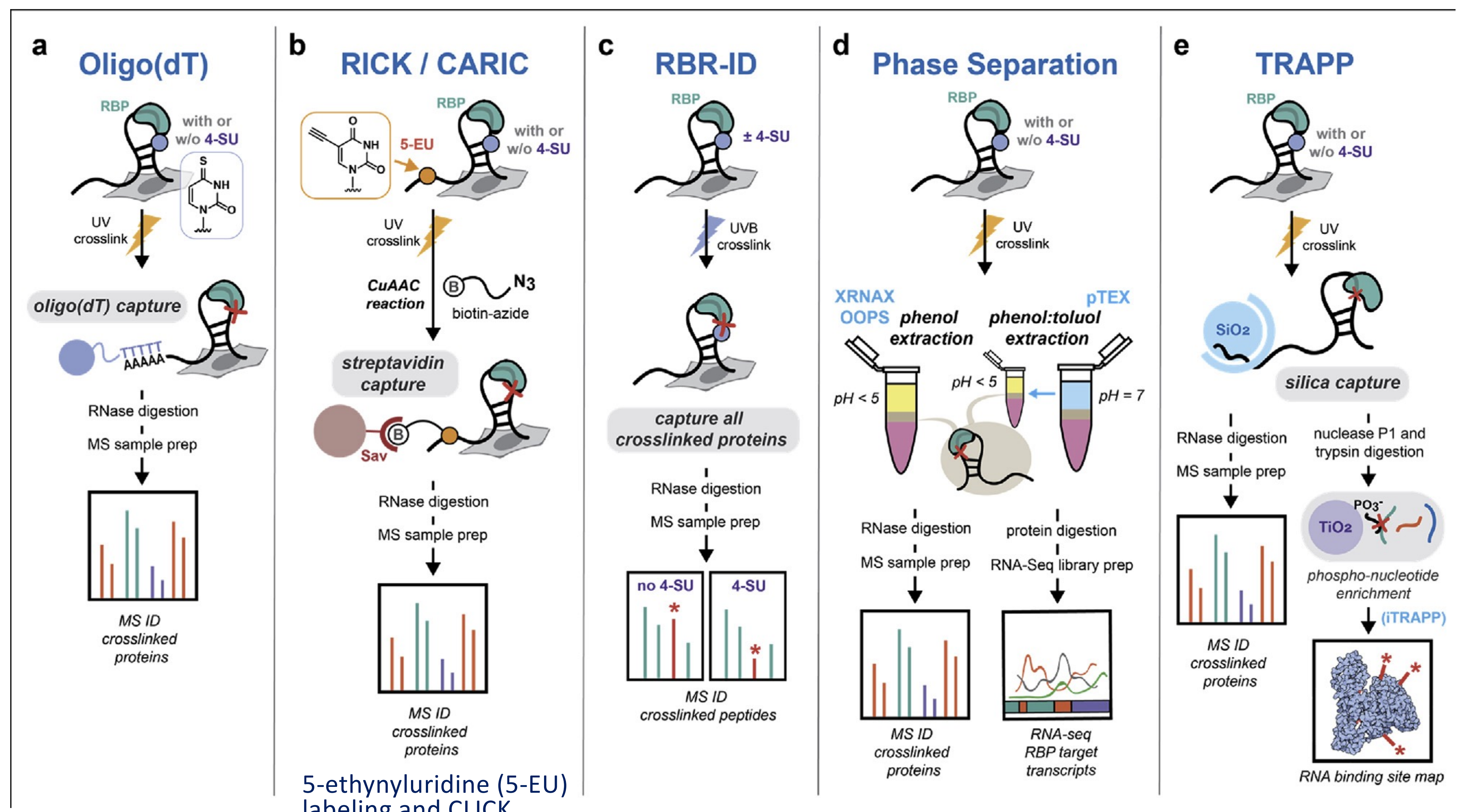
RNA-protein interactions



RNA - protein interactions

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

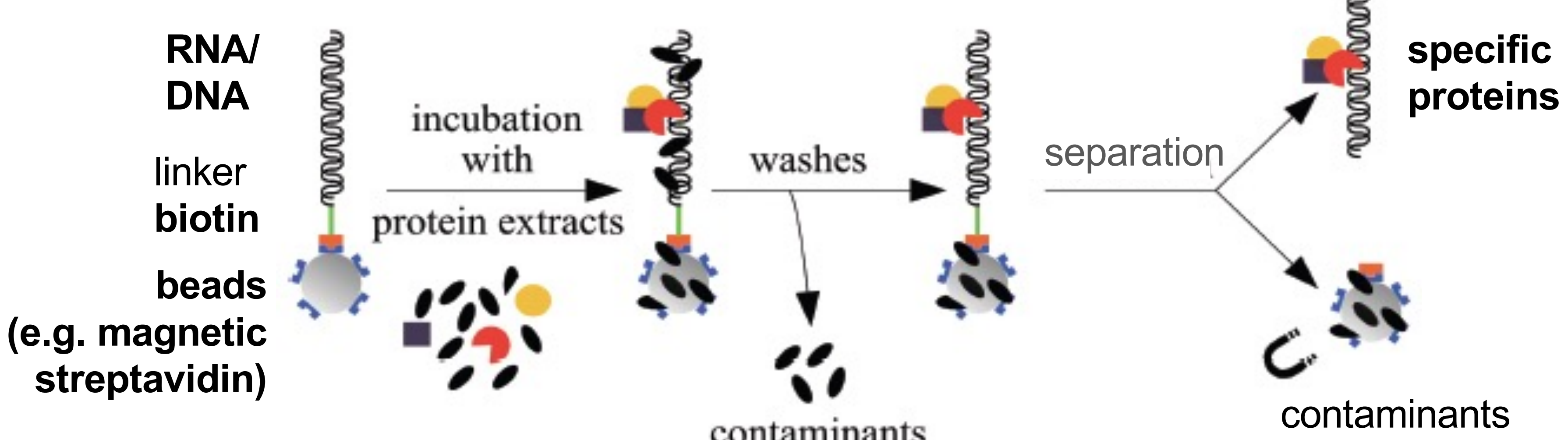
RICK/CARIC: with 5-ethynylU (5-EU), biotin is added to RNA by click chemistry for streptavidin capture



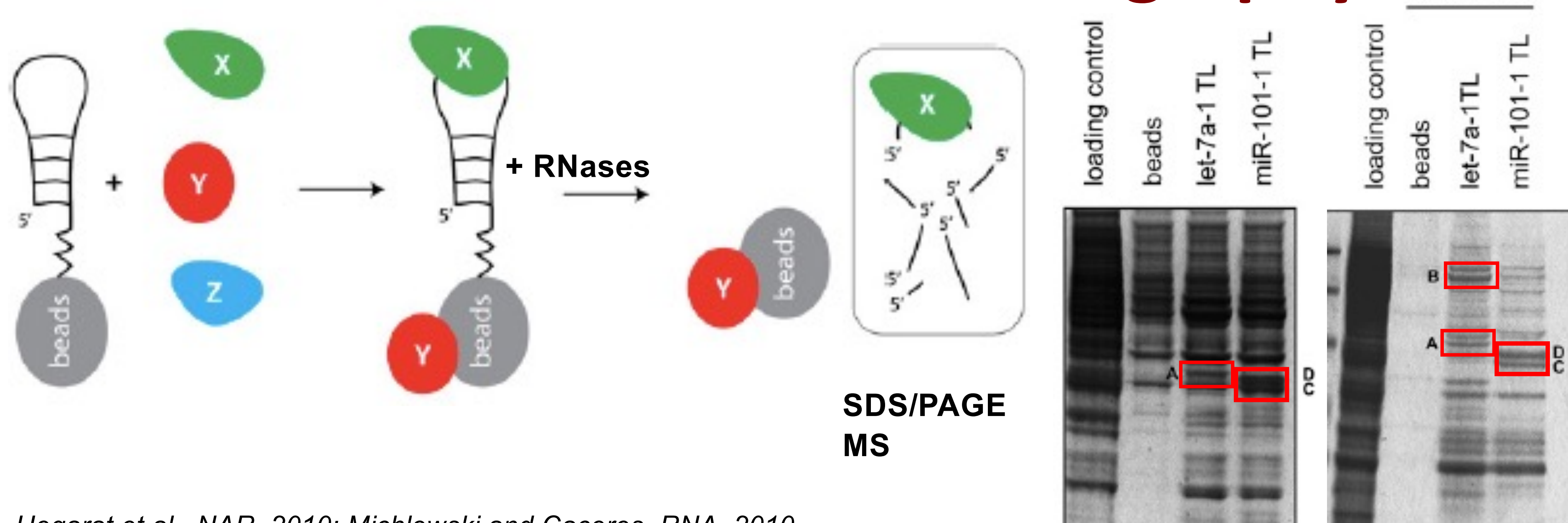
5-ethynyluridine (5-EU) labeling and CLICK chemistry with biotin-azide to enrich RBPs

Nechay and Kleiner, CurrOChem Biol'20

RNA chromatography *in vitro*

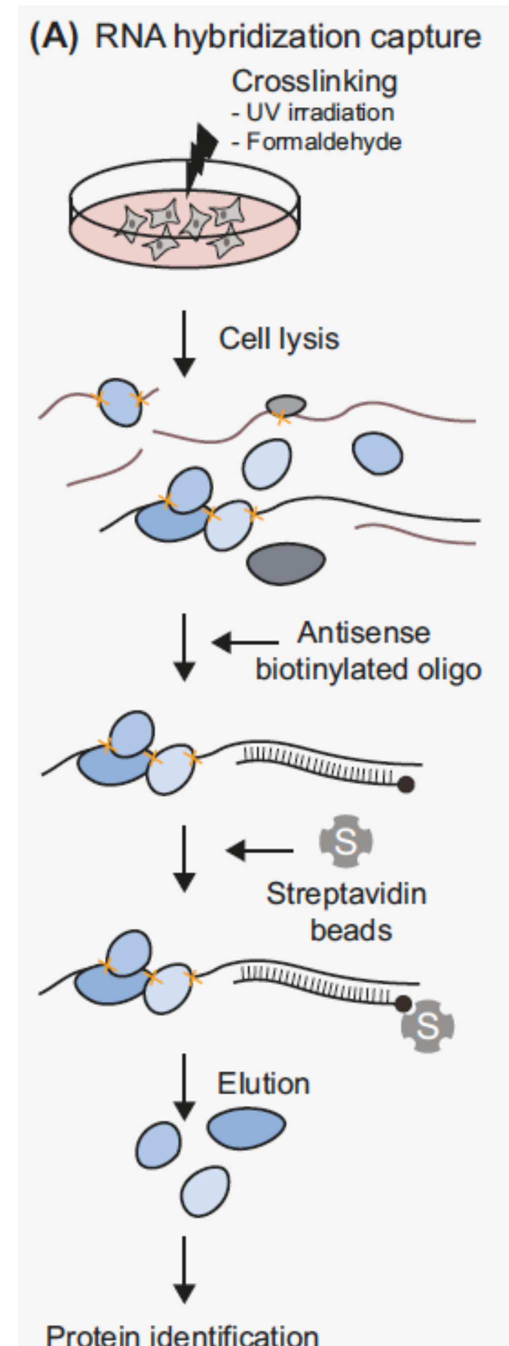


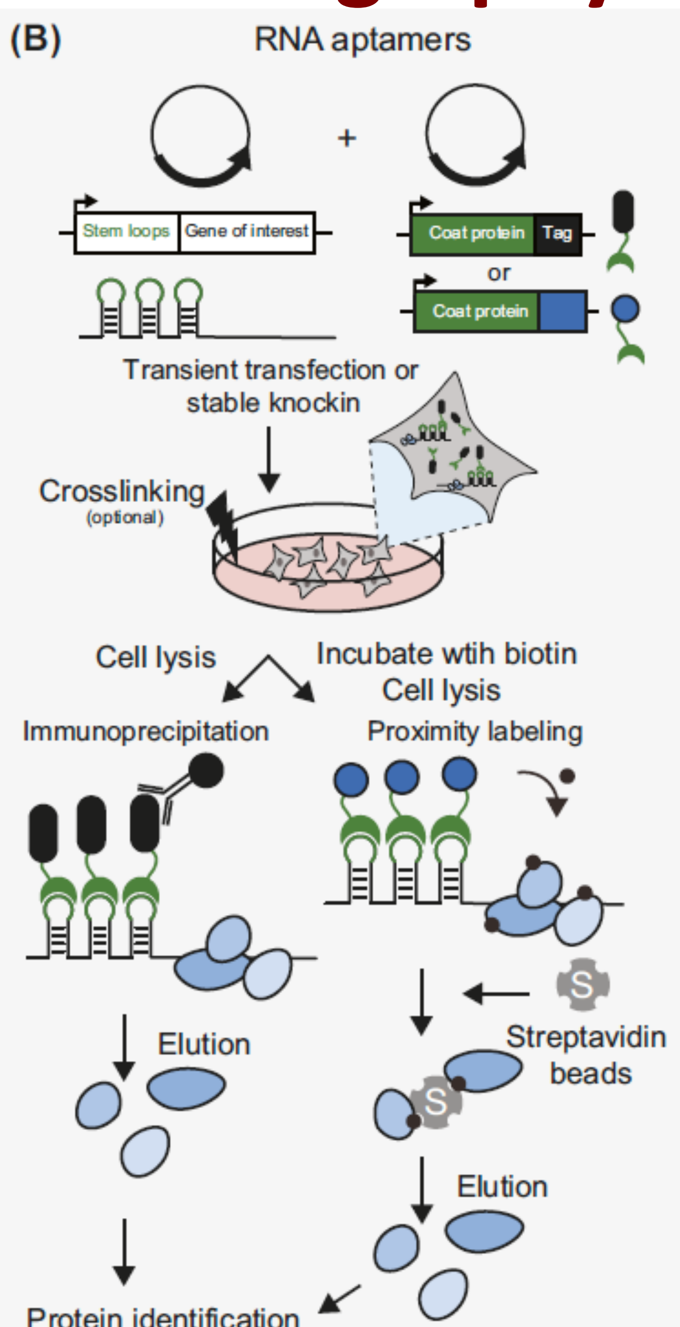
RNase-assisted RNA chromatography

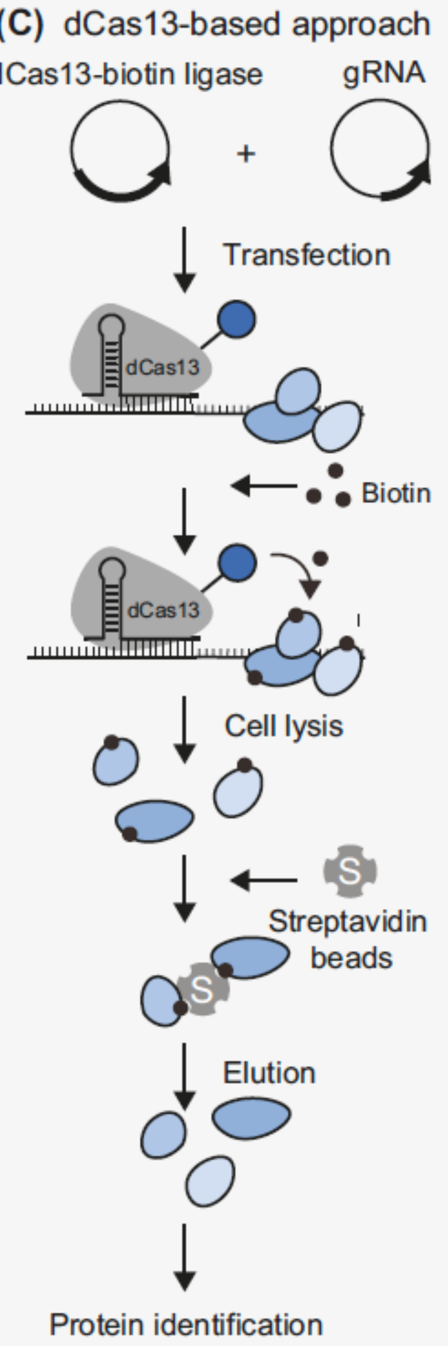


Hegarat et al., NAR, 2010; Michlewski and Caceres, RNA, 2010

RNA chromatography *in vivo*

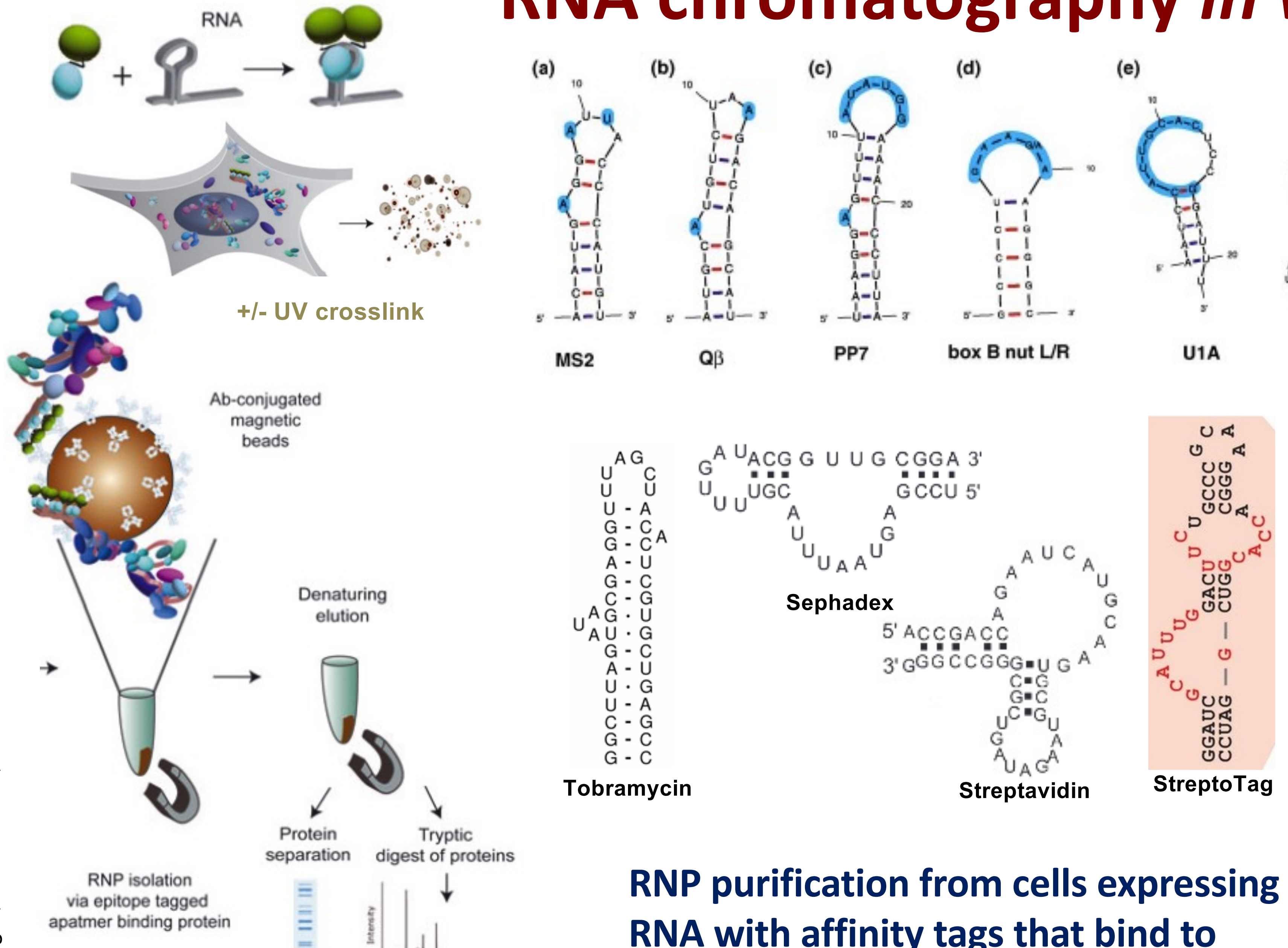






Gräwe et al, *Trends in Biotech* 2021

RNA chromatography *in vivo*



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

RBP

Crosslink

Sonicate

Hybridize

biotinylated tiling oligos

RBP

Purify on bead and wash

streptavidin magnetic beads

RBP

RNase A, H

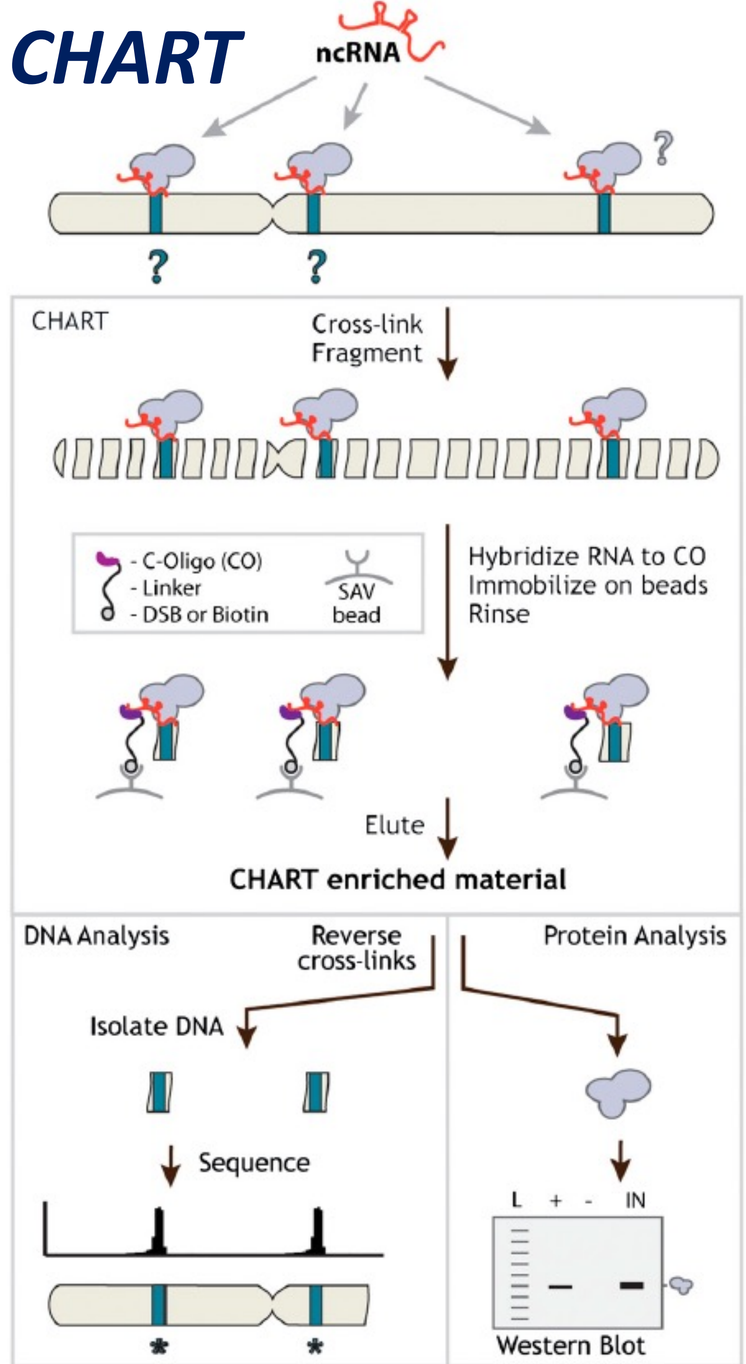
RBP

RNA binding protein

Genomic DNA

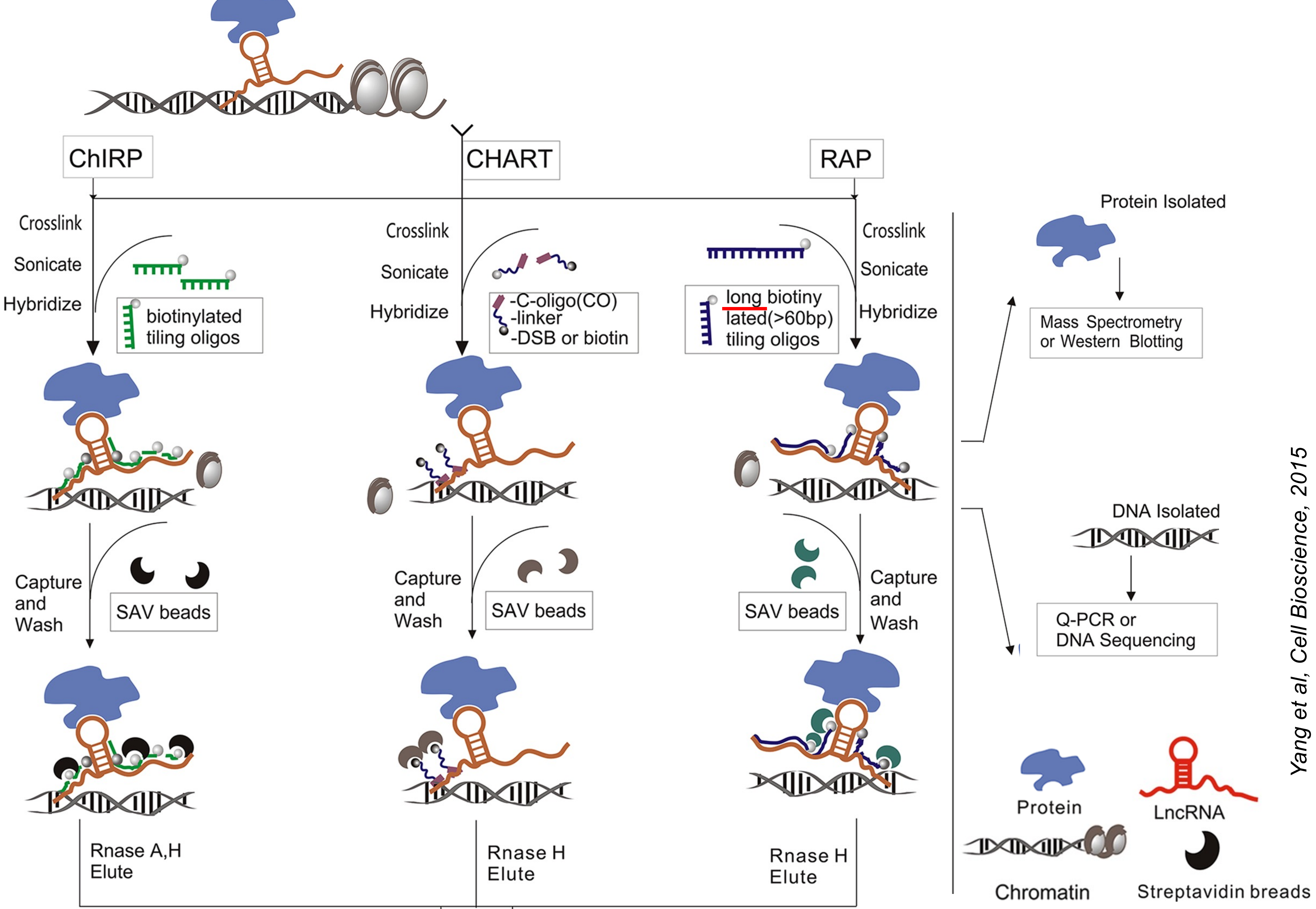
CHART

Capture Hybridization Analysis of RNA Targets

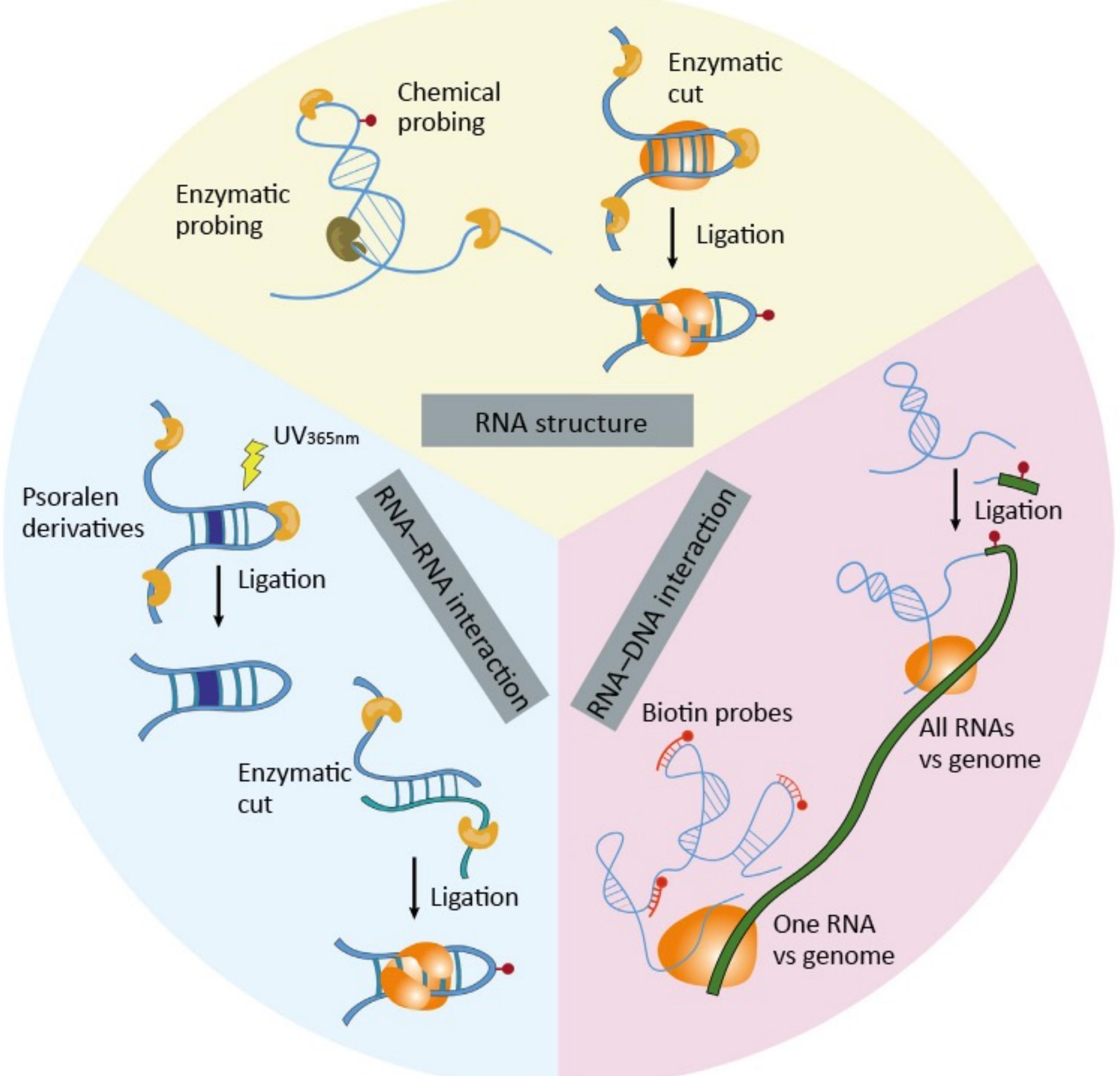


Chu et al., Mol. Cell, 2011; Simon et al., PNAS '11

Interactions lncRNA-proteins-chromatin



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



Nguyen et al, TiG, 2018

RNA structures and RNA-RNA interactions

Ye et al, Mol Cell, 2024

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.	

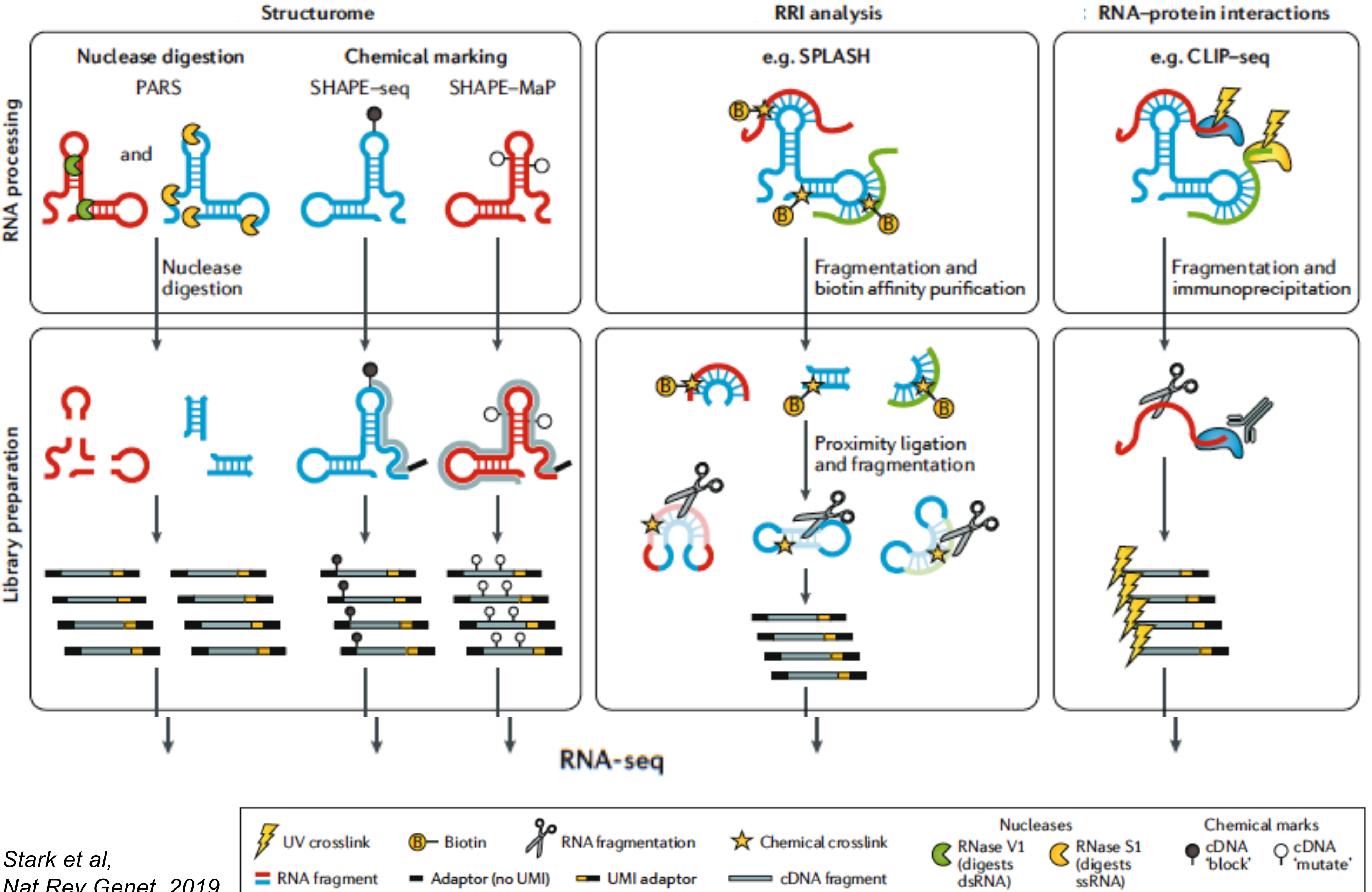
Nguyen et al, TiG, 2018

RNA-seq based mapping of RNA 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 structure and RNA–protein interactions



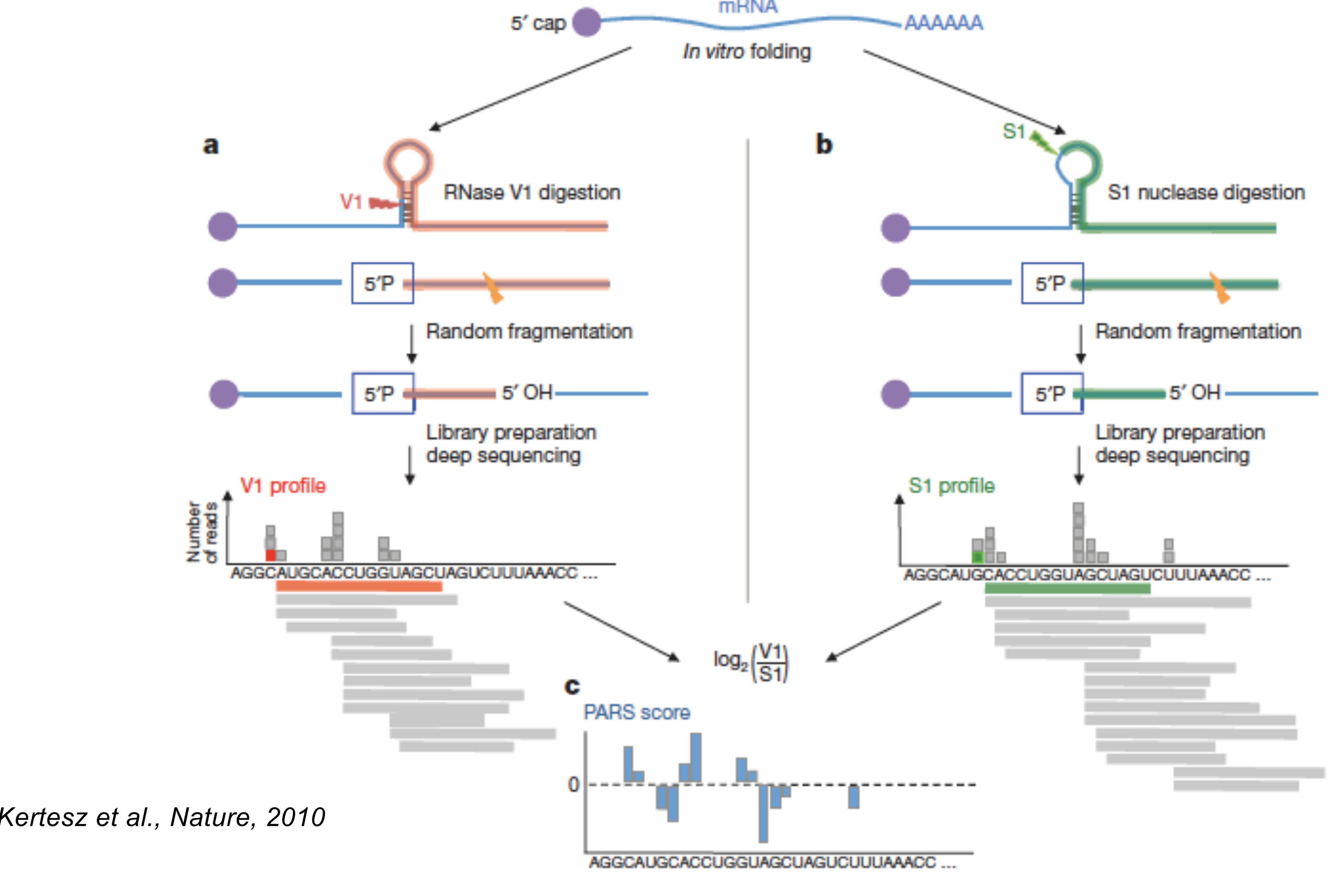
Stark et al,
Nat Rev Genet, 2019

PARS: Parallel Analysis of RNA Structure

RNA structure by enzymatic cleavages followed by RNA-seq

PARS used to establish structures of > 3000 yeast transcripts

Unexpected conclusion: coding mRNA regions are more structured than UTRs



Kertesz et al., Nature, 2010

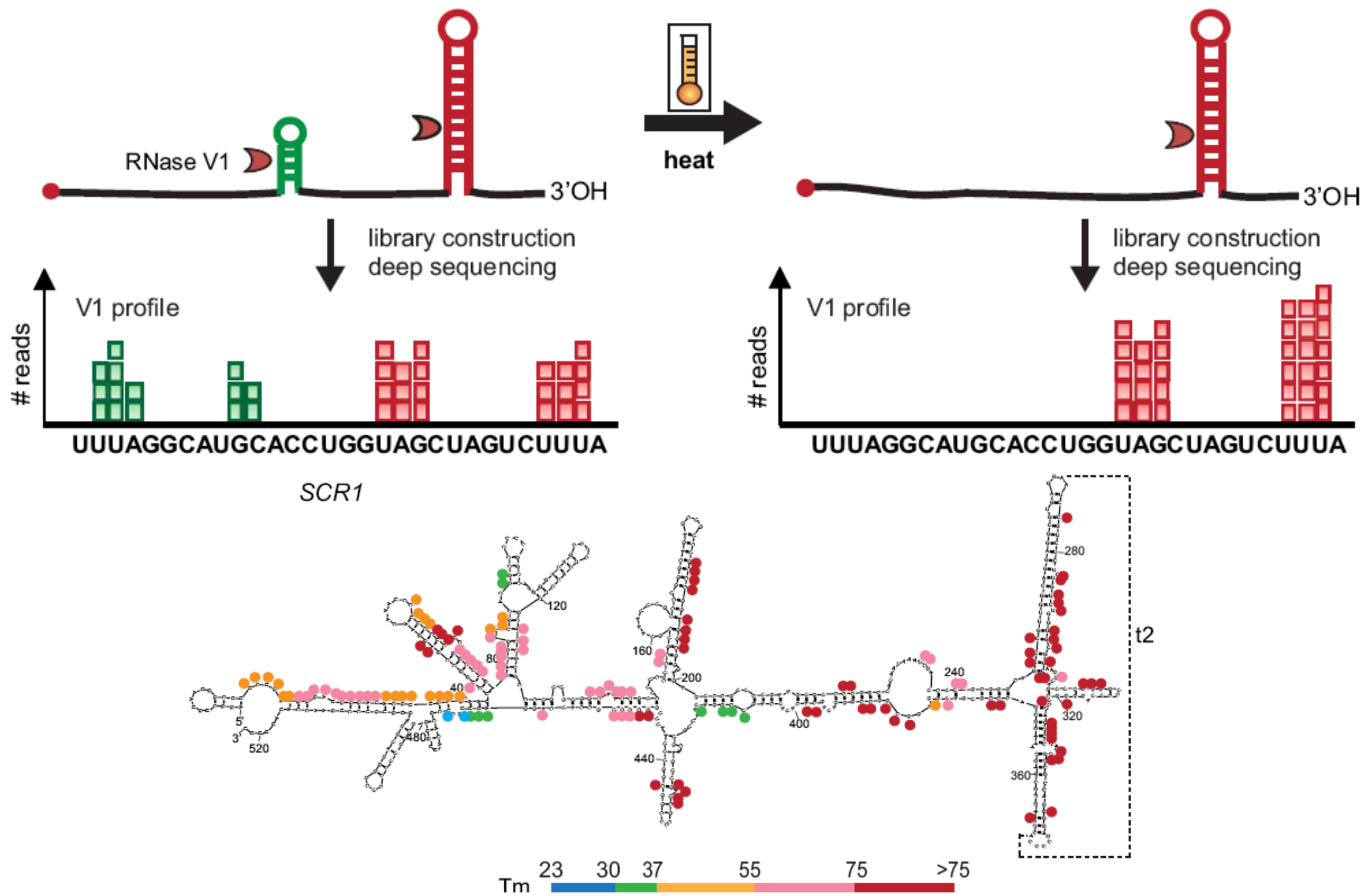
PARS: Parallel Analysis of RNA Structure

Molecular Cell

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,*]

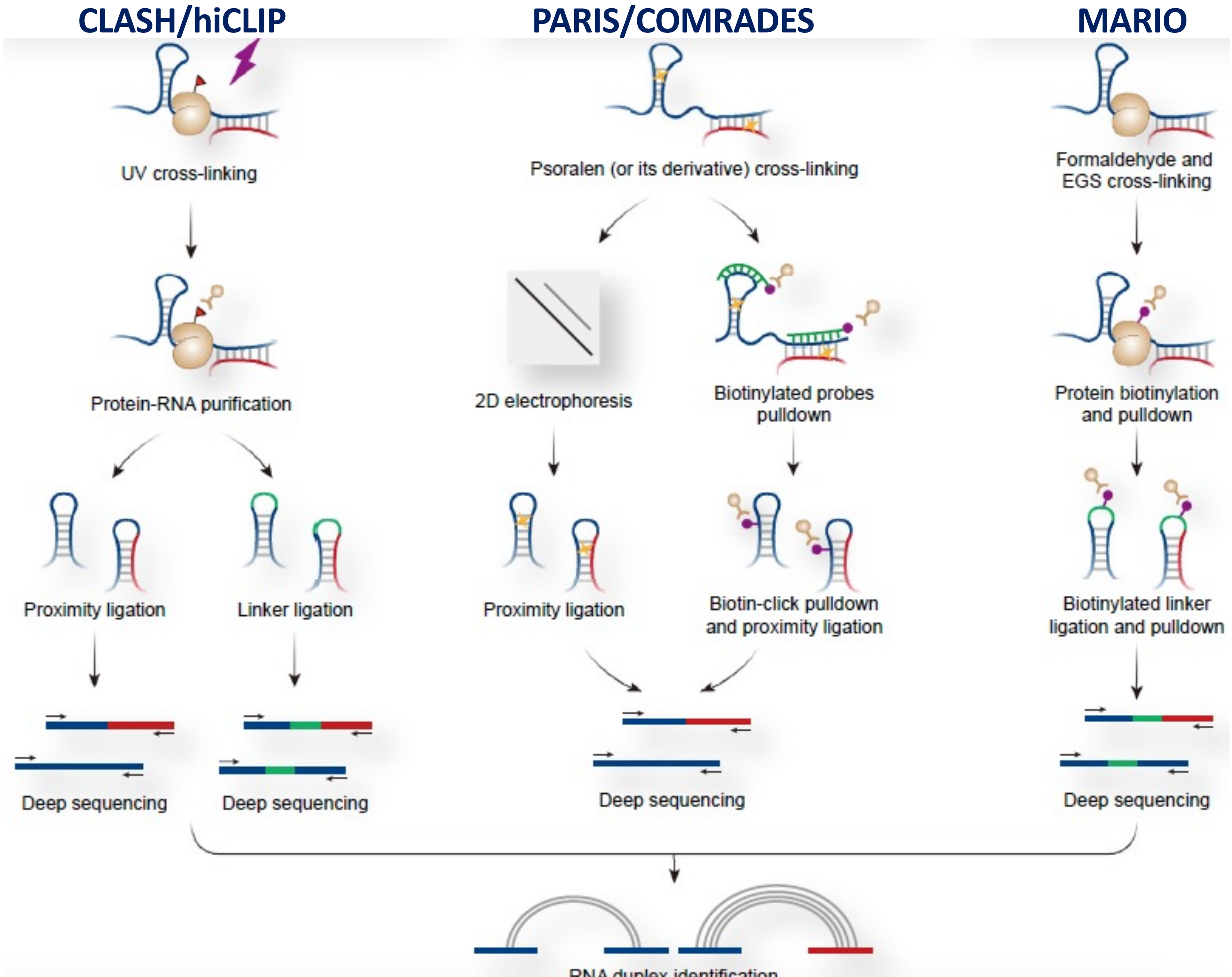


Regulatory Impact of RNA Secondary Structure across the *Arabidopsis* Transcriptome

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]

RNA–RNA interactions

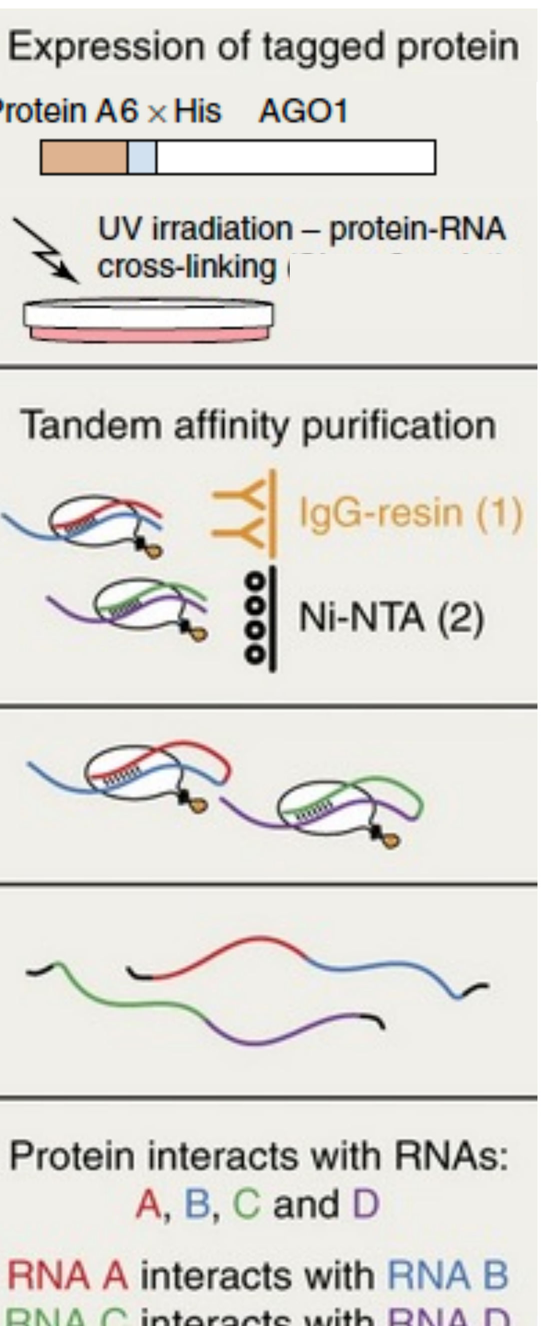


Ye et al, Mol Cell, 2024

CLASH: intra- and intermolecular RNA-RNA interactions

***C**rosslinking*
***L**igation **a**nd*
***S**equencing of*
***H**ybrids*

> 99 %
< 1 %
RNA A
RNA B
RNA A RNA B

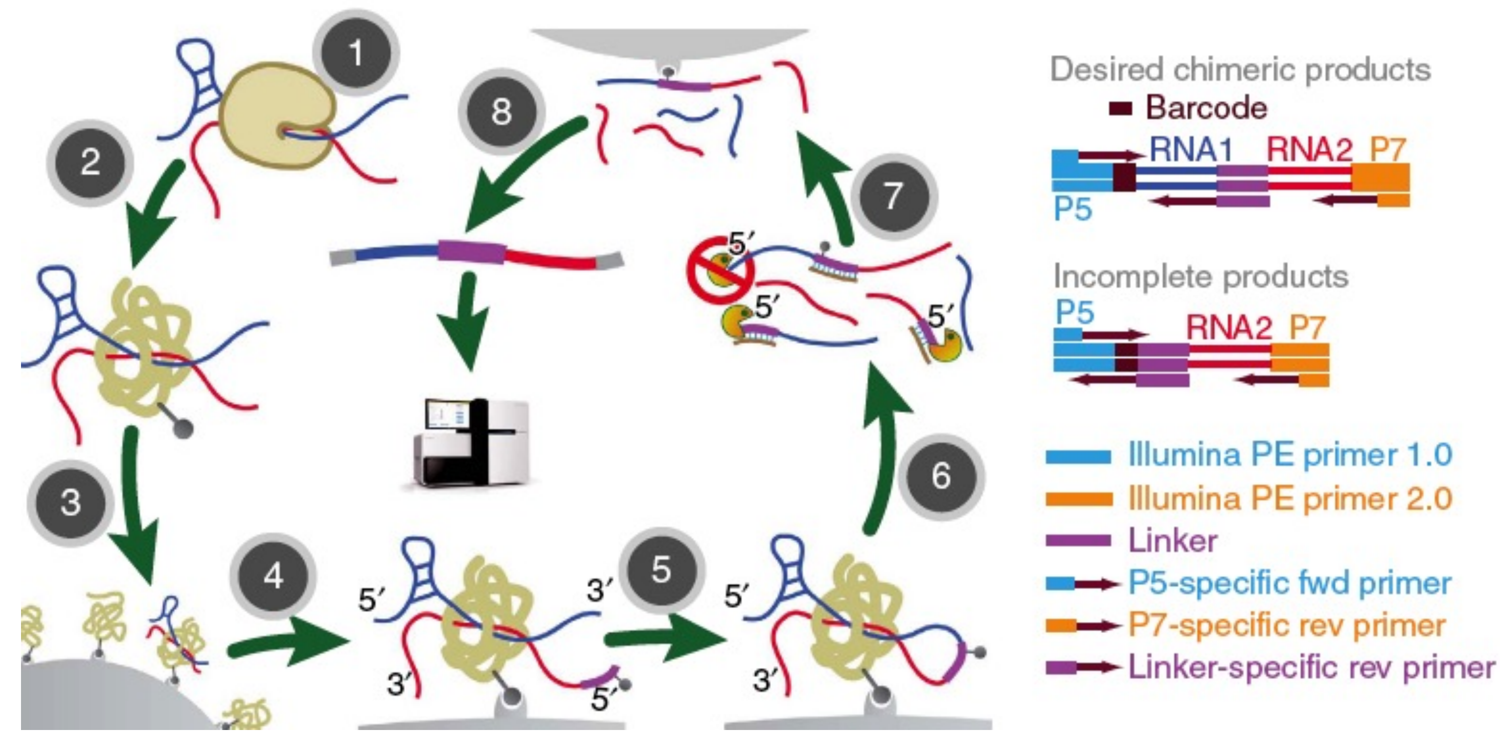


Kudla et al., PNAS, 2011; Helwak and Tollervey, Nat Protocol 2014

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

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

Chemical and enzymatical - based structure probing

SHAPE: Selective 2'- Hydroxyl Acylation and Primer Extension; **SHAPE-seq:** SHAPE and 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

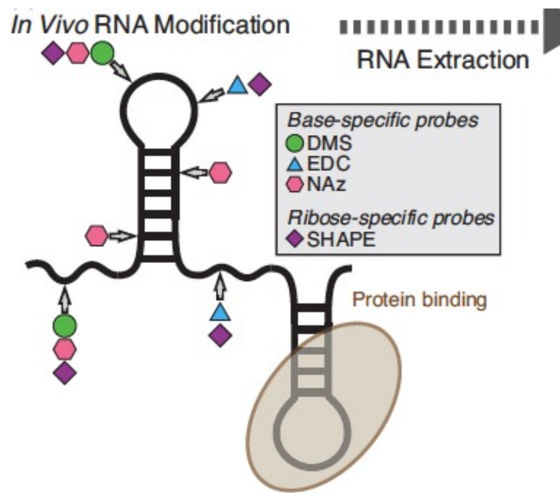
SHAPE chemicals: DMS, dimethyl sulfate; 1M7, 1-methyl-7-nitroisatoic anhydride
SHAPE enzymes: P1 nuclease, RNases V1 and S1
PARIS/SPLASH chemicals: psoralen; AMT, 4'-aminomethyltrioxsalen

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,



Reverse Transcription

RT Stop Analysis (Structure-seq, LASER-seq, icSHAPE)

3' 5' cDNA
5' 3' RNA
Base or sugar modification

Mutational Profiling (MaPseq, LASER-MaP, SHAPE-MaP)

cDNA mutation
3' 5' cDNA
5' 3' RNA
Base or sugar modification

Library Preparation → Next-Gen Sequencing → Structure Prediction

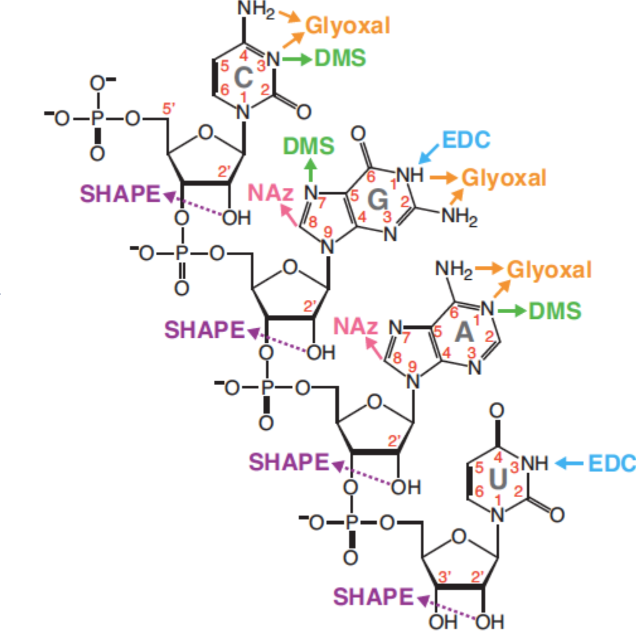
	Probe	Primary modification sites
SHAPE	N-methylisatoic anhydride (NMIA)	2′OH of all nts
	1-methyl-7-nitroisatoic anhydride (1M7)	2′OH of all nts
	1-methyl-6-nitroisatoic anhydride (1M6)	2′OH of all nts
	Benzoyl cyanide (BzCN)	2′OH of all nts
	2-methylnicotinic acid imidazolide (NAI)	2′OH of all nts
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

sugar

base

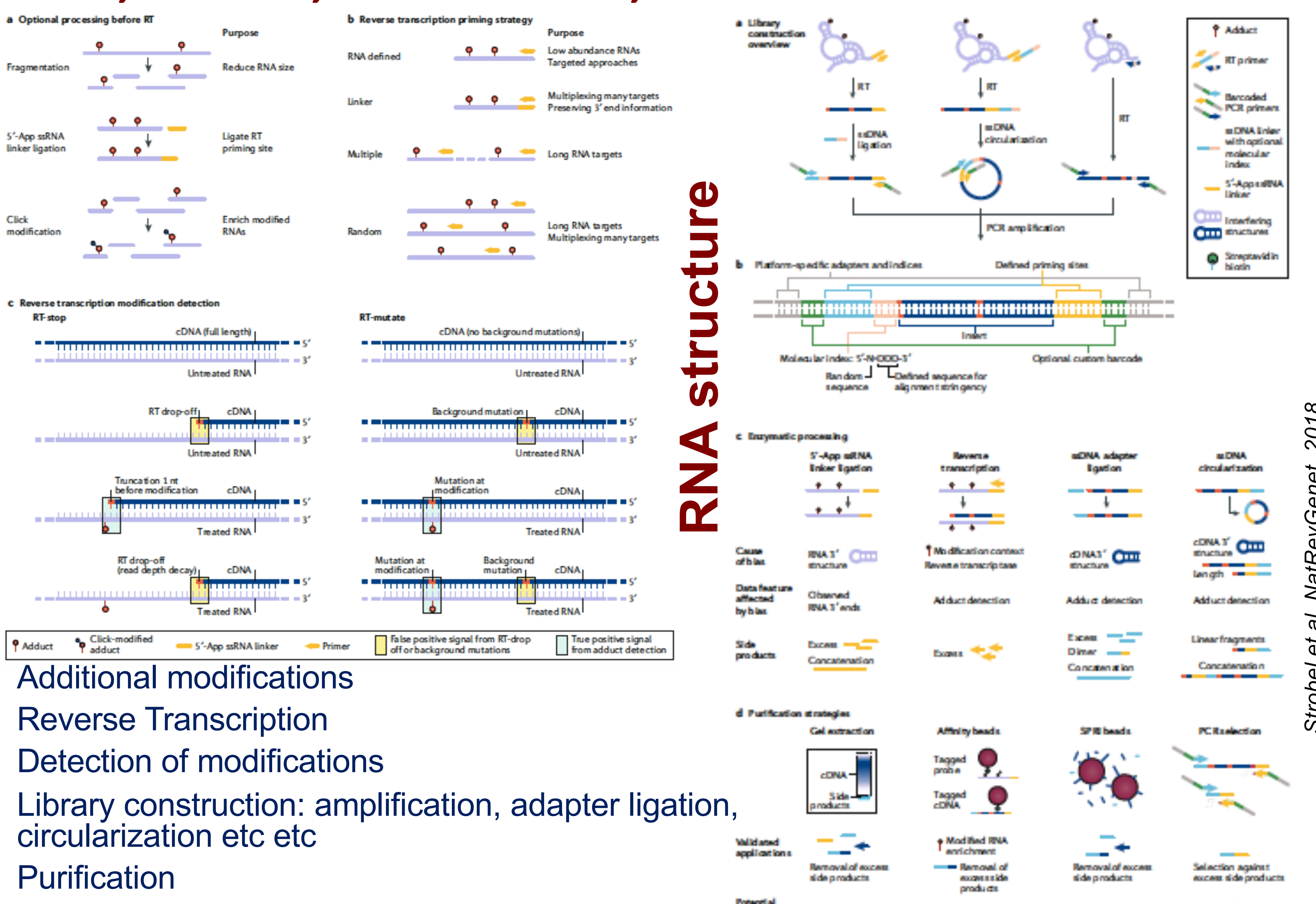
sugar

base



Mitchel III et al, Curr Op Struct Biol, 2019
Strobel et al, Nat Rev Genet, 2018

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



Additional modifications

Reverse Transcription

Detection of modifications

Library construction: amplification, adapter ligation, circularization etc etc

Purification

Structure calculation

RNA structure
PARIS, SPLASH, LIGR

PARIS

AMT +
365 nM Irradiation

Proteinase
RNase
2D Gel Purification

Proximity Ligation

Reverse Crosslink

Library Preparation
Sequencing

LIGR-Seq

AMT +
365 nM Irradiation

rRNA depletion
S1 digestion

Denature
circRNA ligase

RNaseR
Reverse Crosslink

Library Preparation
Sequencing

SPLASH

Bio-Psoralen +
365 nM Irradiation

RNA Extraction
Fragmentation
Bead Enrichment

Proximity Ligation

Reverse Crosslink

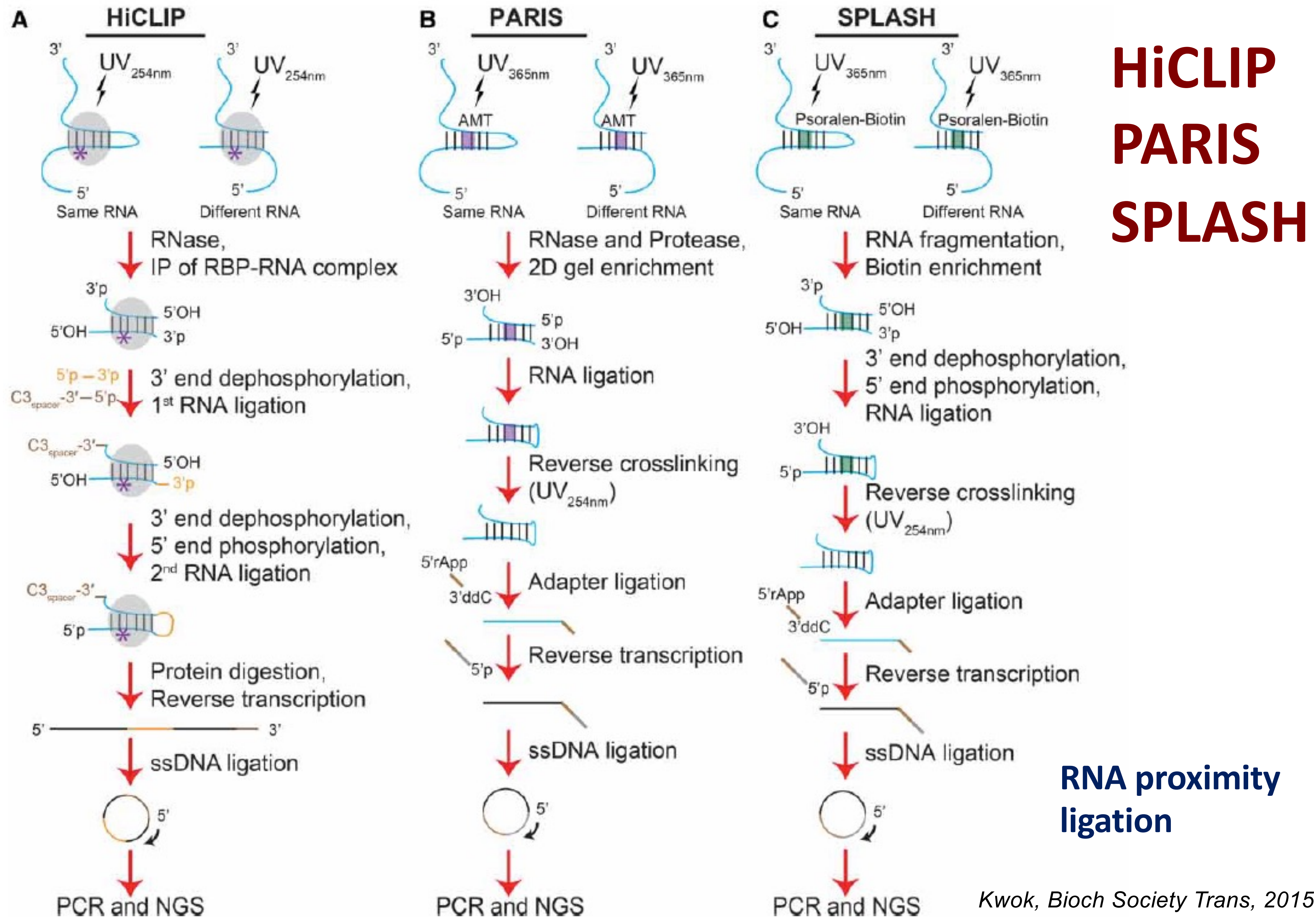
Library Preparation
Sequencing

- *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-protein interactions and RNA structure



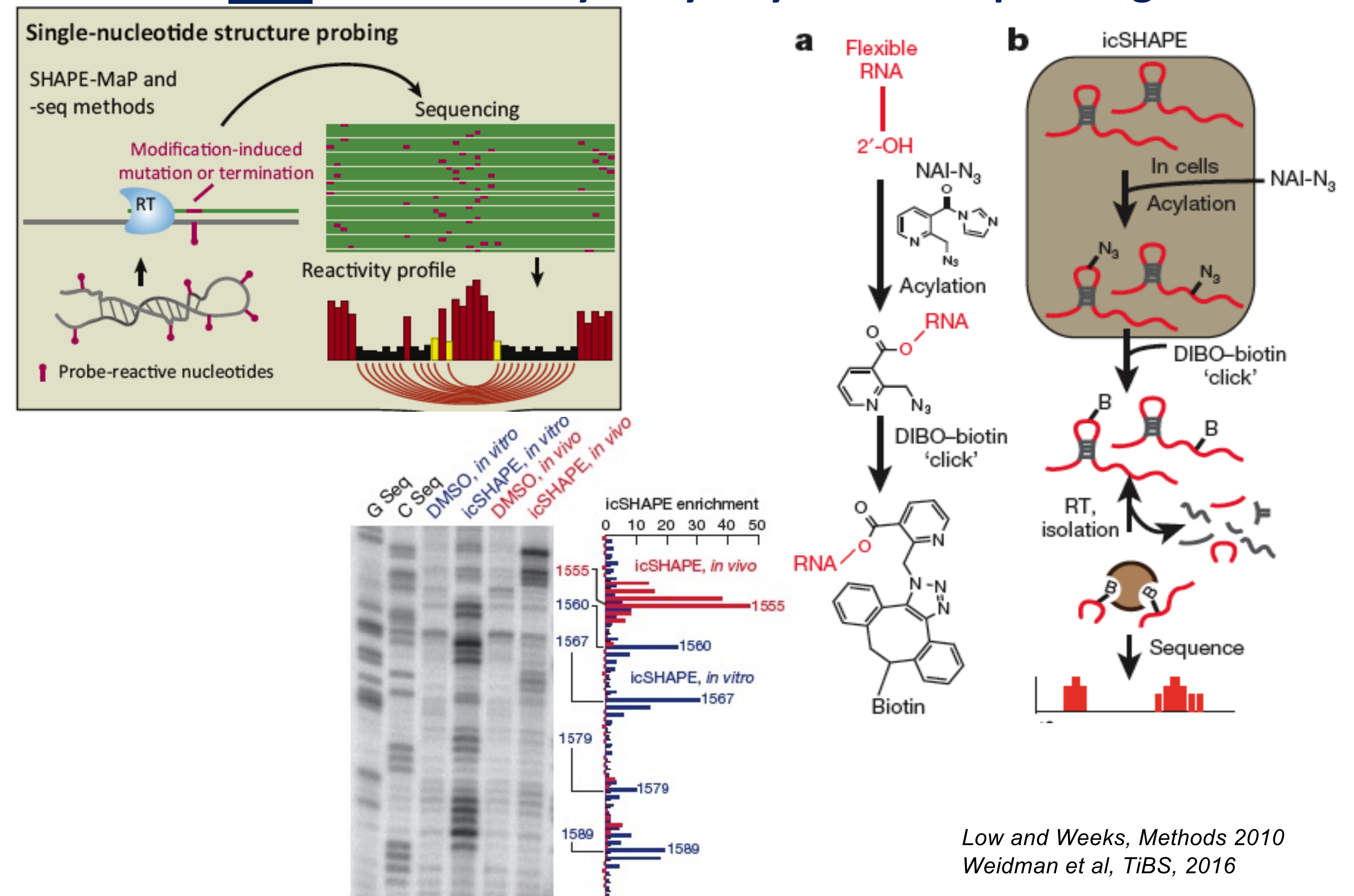
HiCLIP
PARIS
SPLASH

RNA proximity ligation

Kwok, Bioch Society Trans, 2015

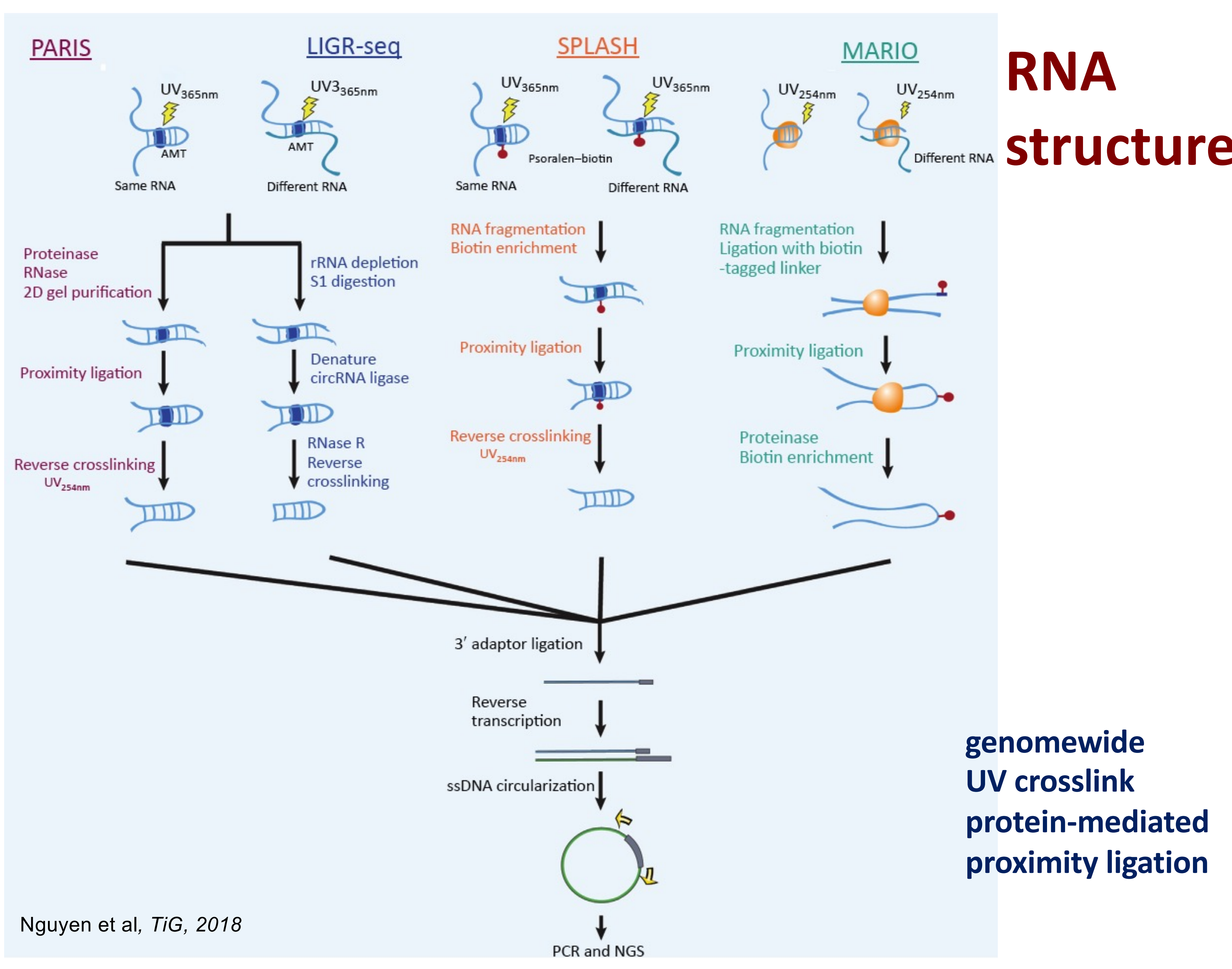
icSHAPE: RNA structure *in vivo*

icSHAPE: click selective 2'-hydroxyl acylation and profiling



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

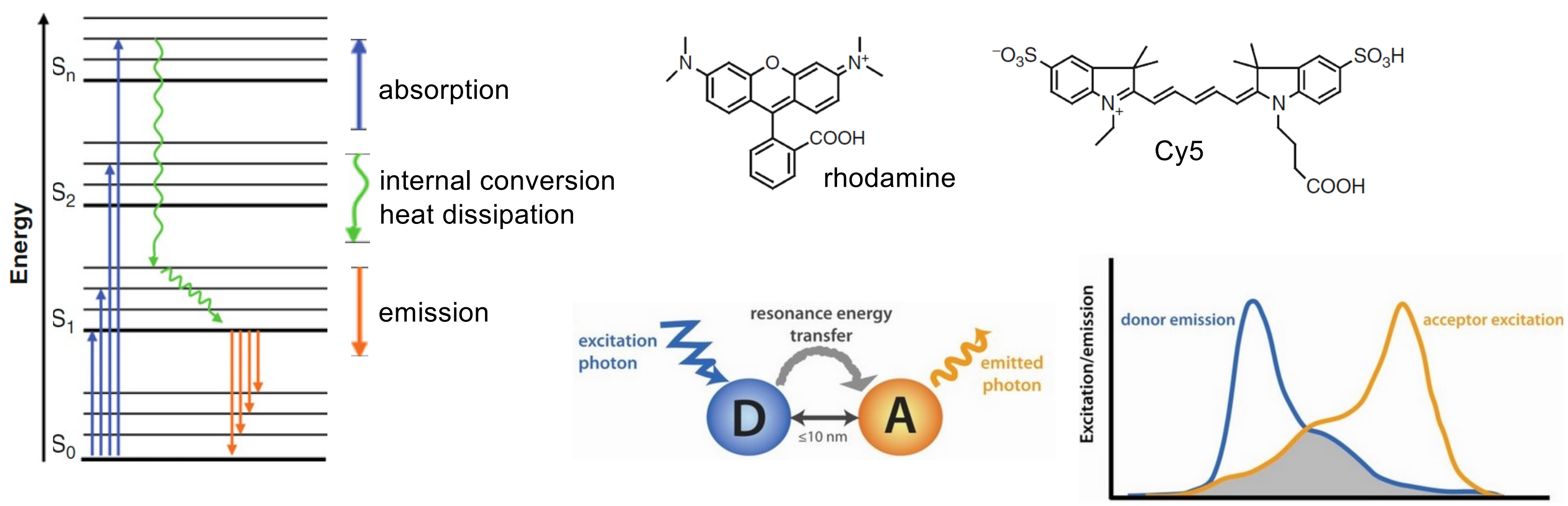
RNA structure



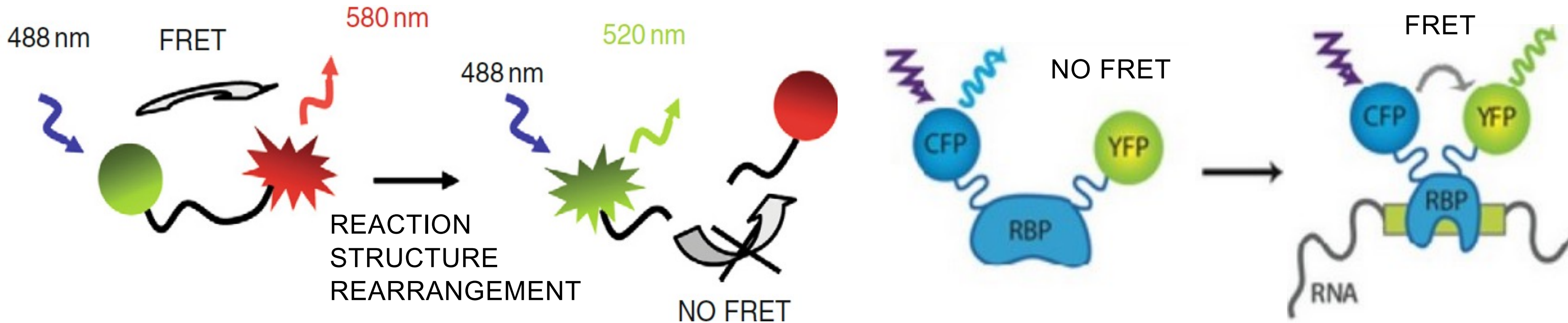
genomewide
UV crosslink
protein-mediated
proximity ligation

mRNA fluorescent labeling

FRET: fluorescent (Förster) resonace energy transfer

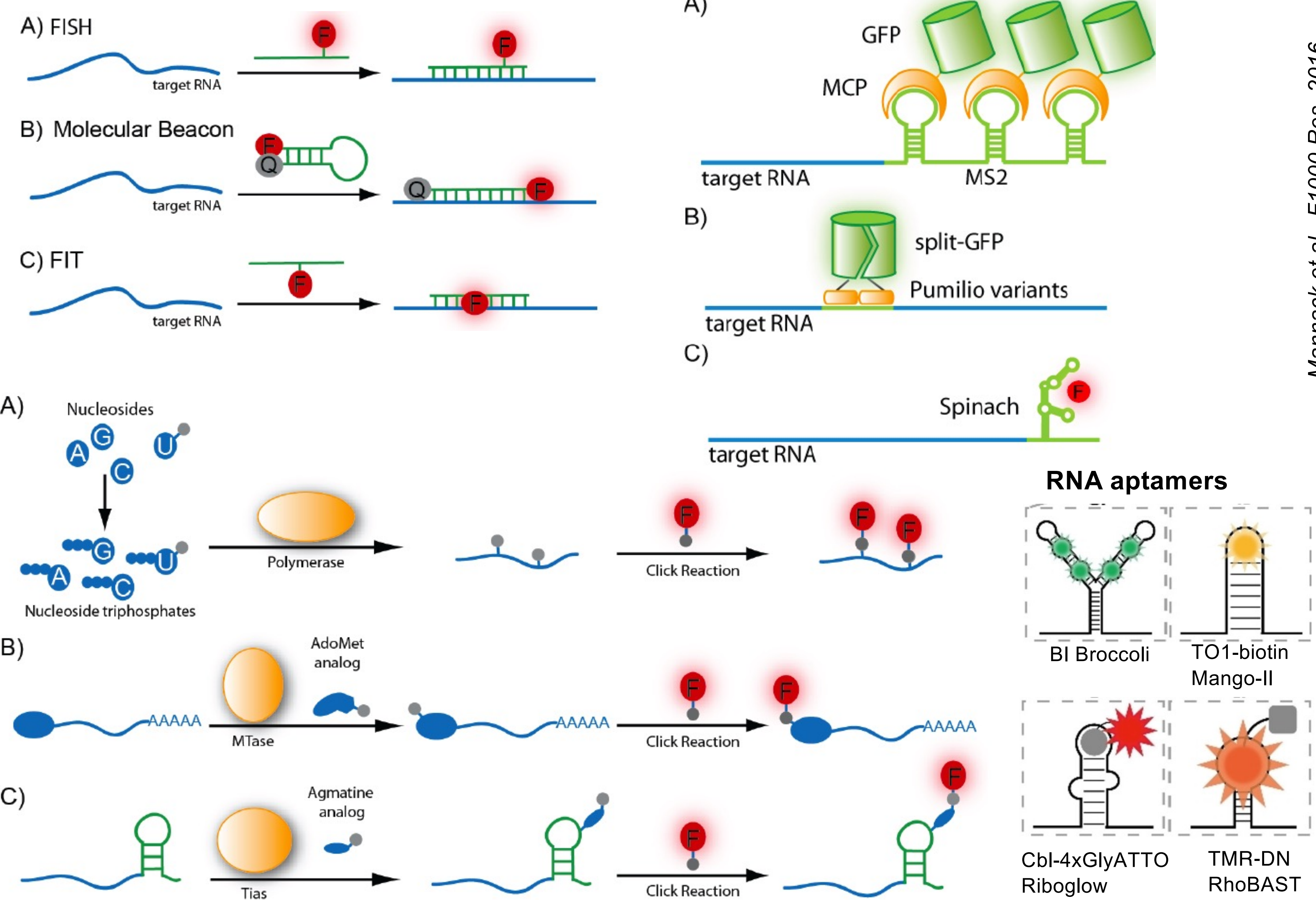


RNA modifications (cleavage, ligation, etc)
RNA structure remodeling
RNA-RNA, RNA-protein and protein-protein interactions



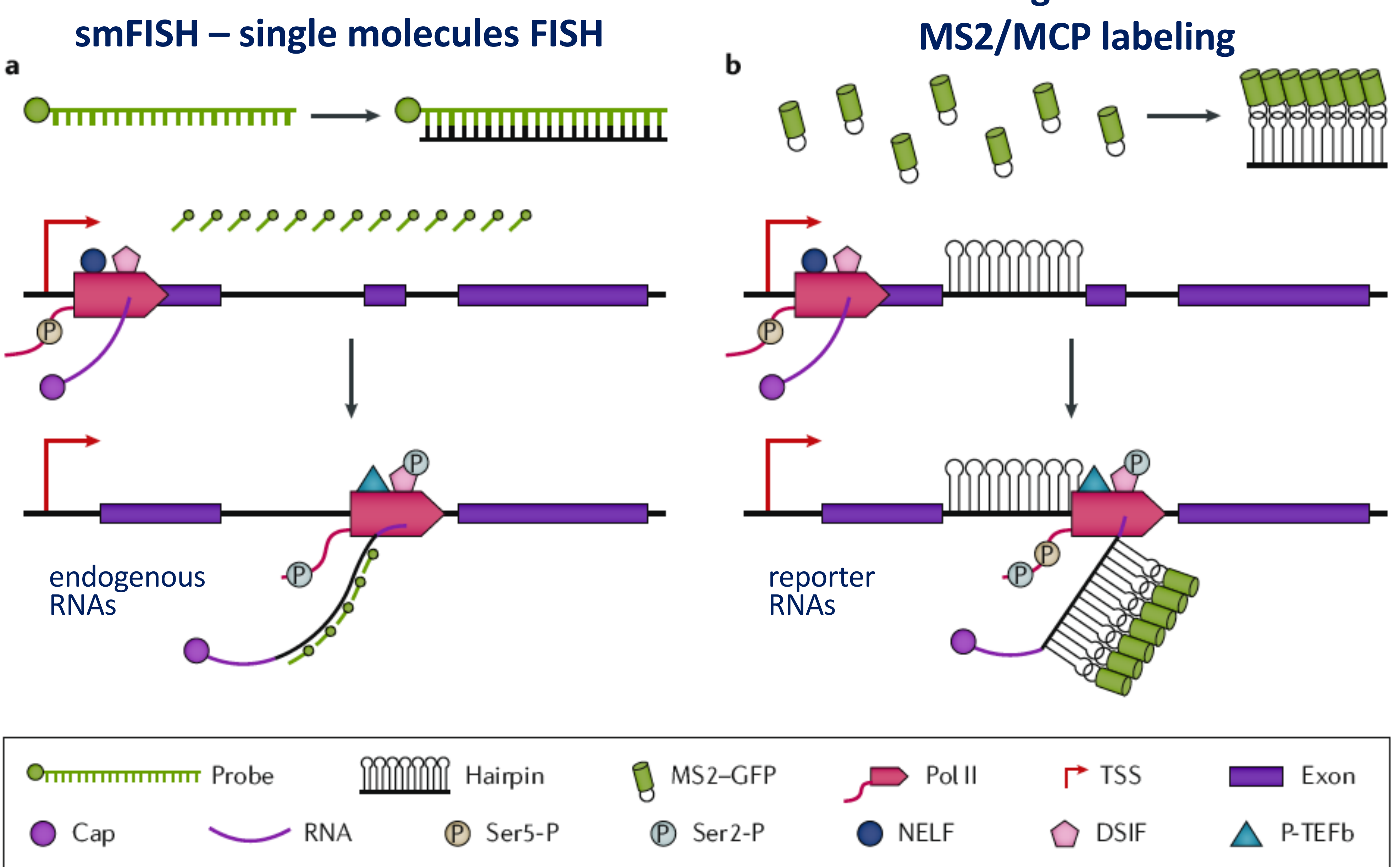
Correa and Schults, Lab Yechniques, 2009

RNA fluorescent labelling for imaging



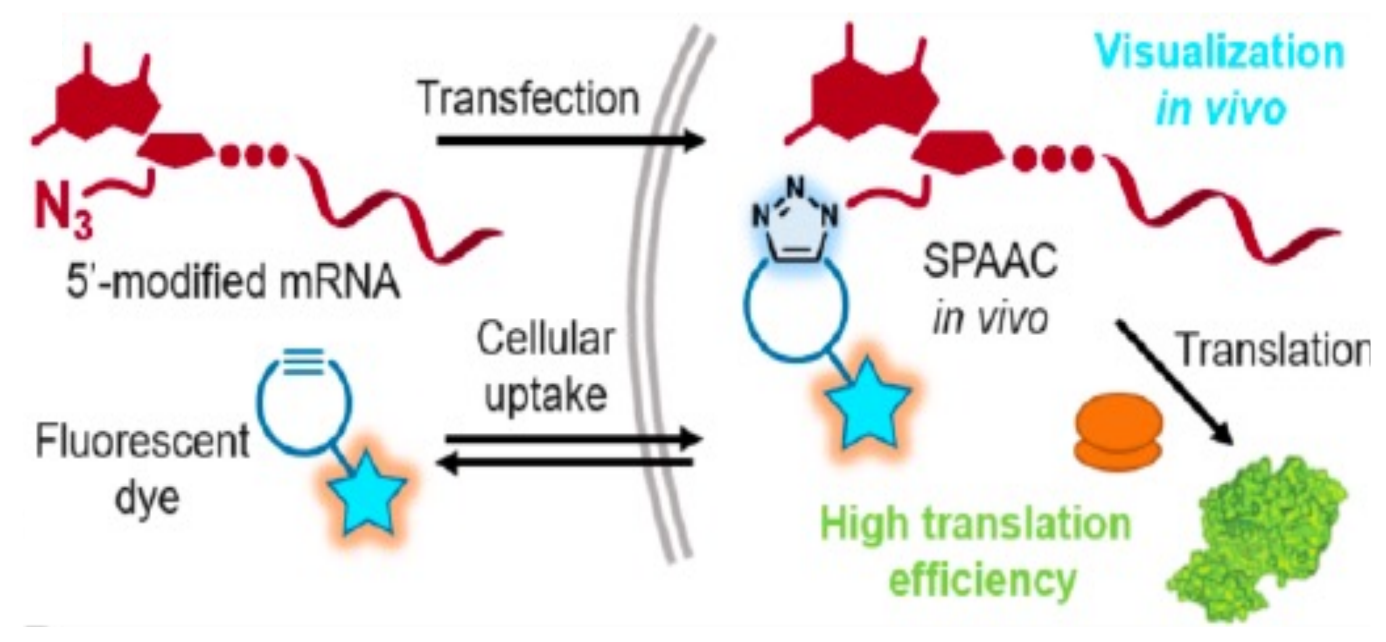
Imaging of nascent RNA

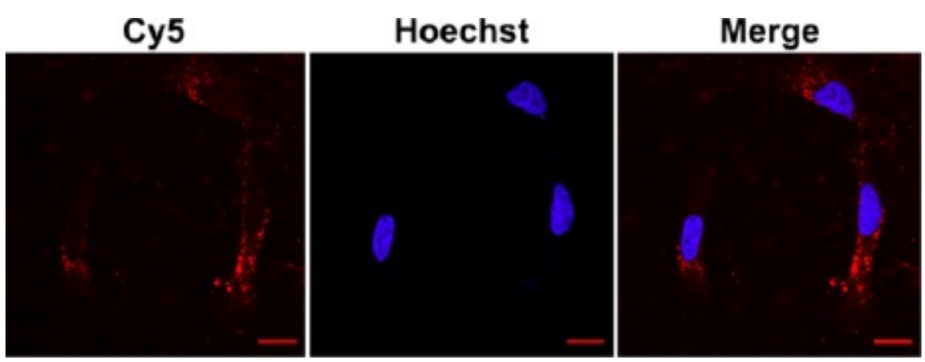
in living cells



Wissink et al, Nat Rev Genet, 2019

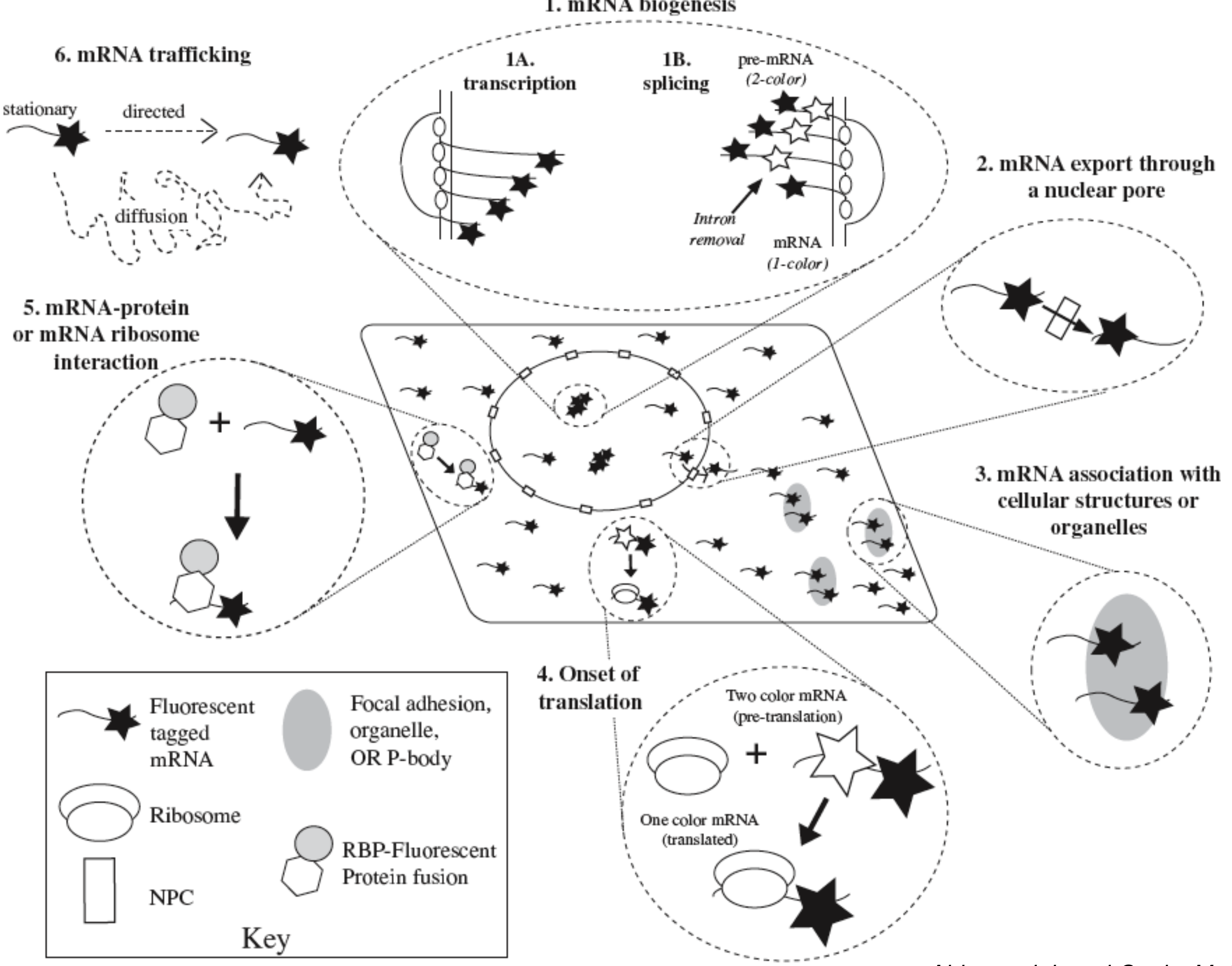
RNA fluorescent labelling for imaging





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

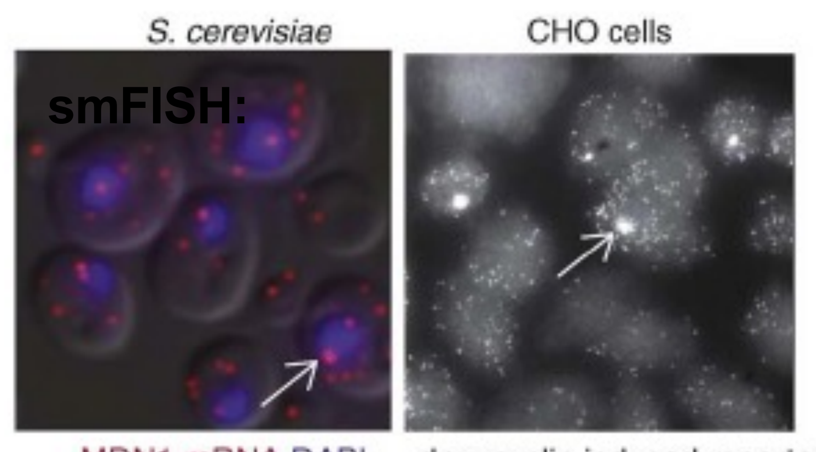
FISH: Fluorescent *in situ* hybridization



Abbaszadeh and Gavis, Methods, 2016

RNA localization

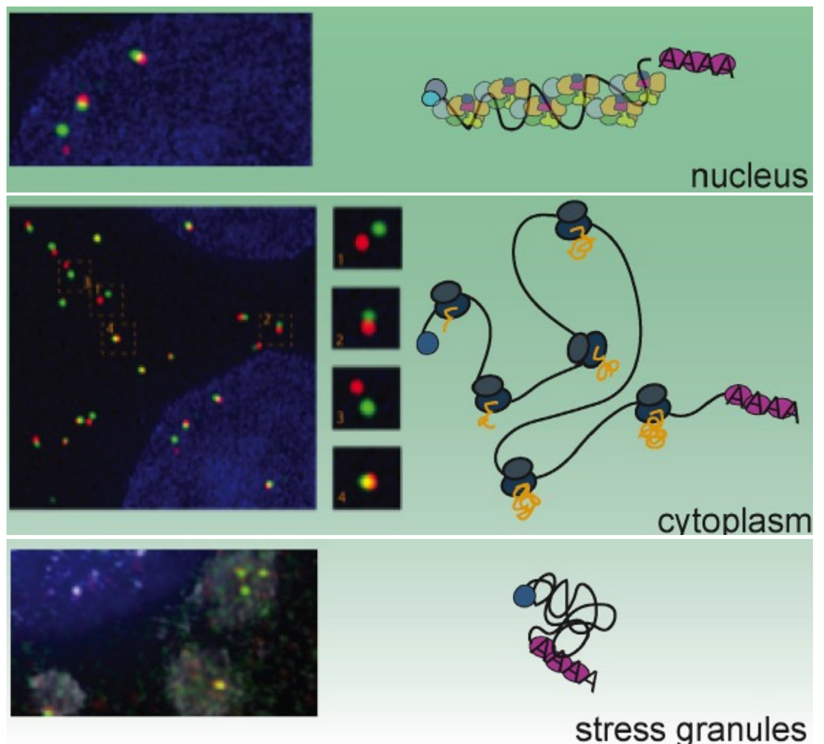
Complex quantification allows analysis of single-molecule gene expression, e.g. transcription/splicing in real time, RNA level in single cells.



Larson et al., TiCB, 2009

at transcription sites (nucleus)

Multi-colored smFISH:



Adivarahan et al., Mol Cell 2018

nucleus, cytoplasm and SG

Constitutively expressed genes are transcribed by single events separated in time.
Regulated genes (e.g. by SAGA) are expressed by transcriptional bursts

Nuclear mRNAs are partially extended
Translating mRNAs usually do not have a stable circular form
mRNAs in stress granules are more compacted than translating mRNAs

FRAP and FLIP: RNA localization

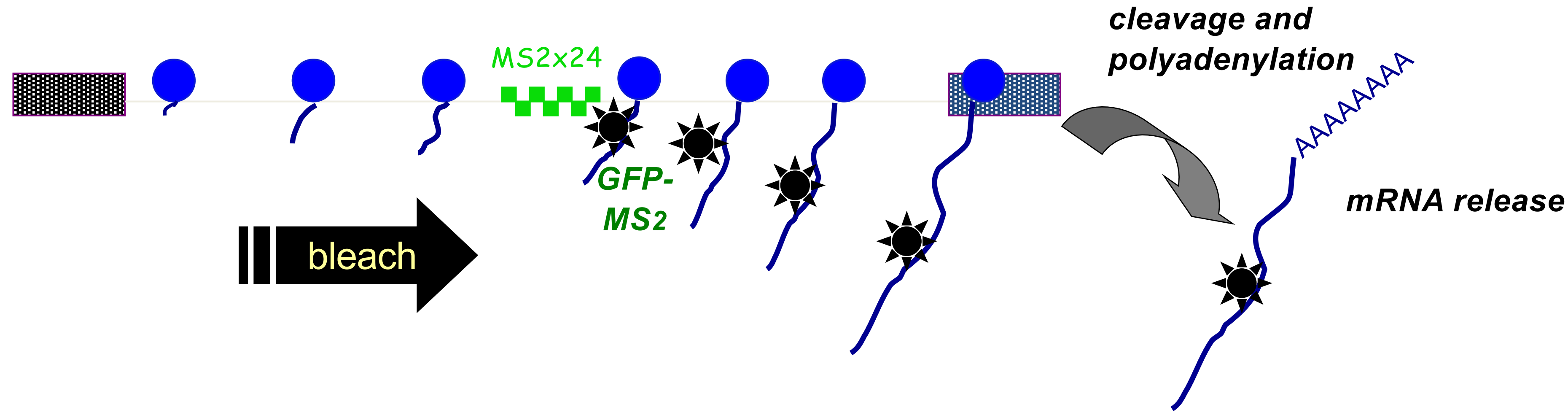
Analysis of molecule kinetics in living cells

FRAP - Fluorescence Recovery After Photobleaching

FLIP - Fluorescence Loss In Potobleaching

Edouard Bertrand, Montpellier, *RIBOSYS*

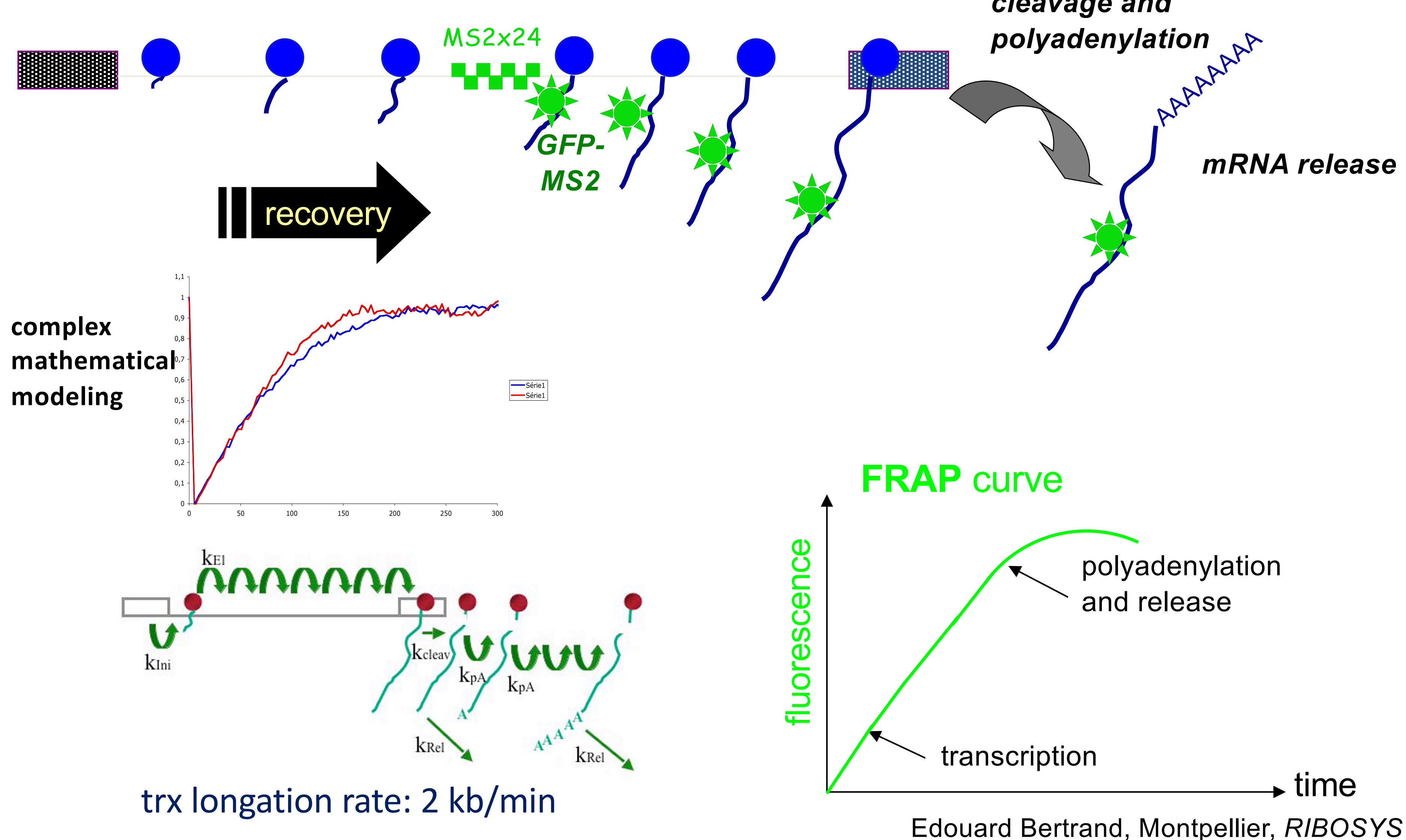
FRAP: RNA localization



Edouard Bertrand, Montpellier, *RIBOSYS*

FRAP: RNA localization

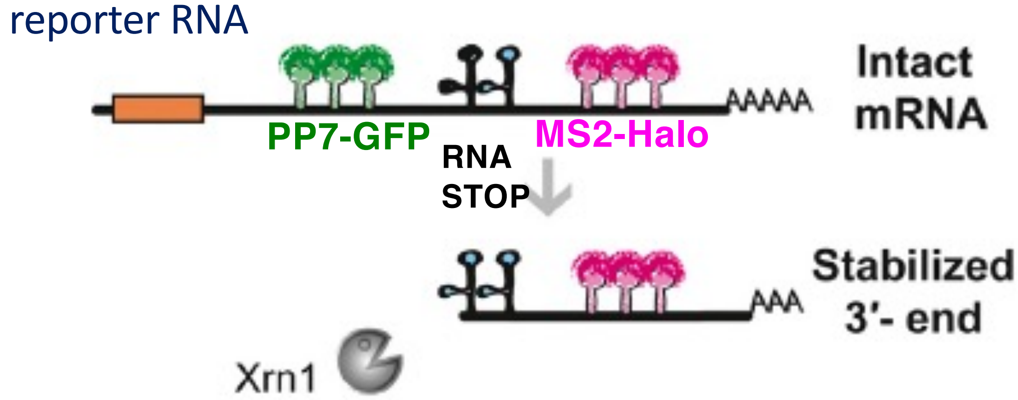
Analysis of:
- **transcription rates**
- **3'-end formation**
- **transcript release**

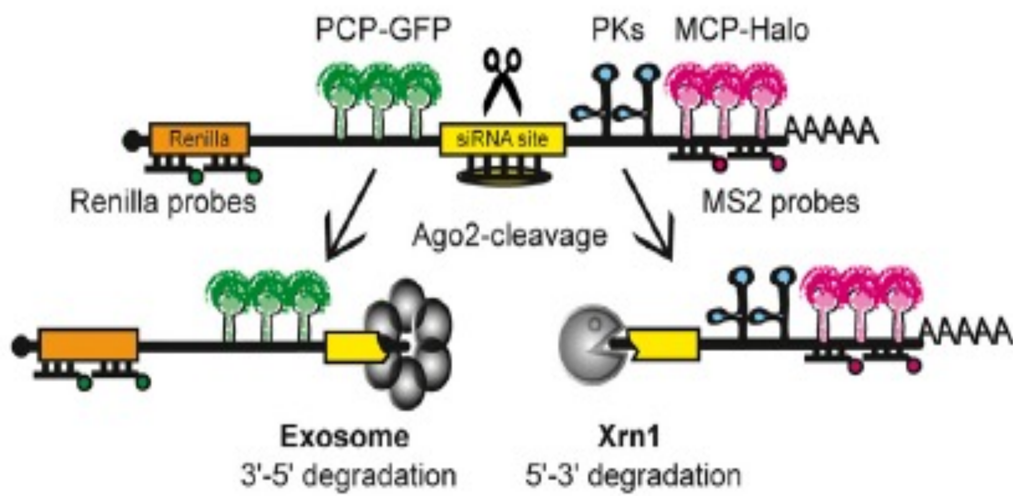


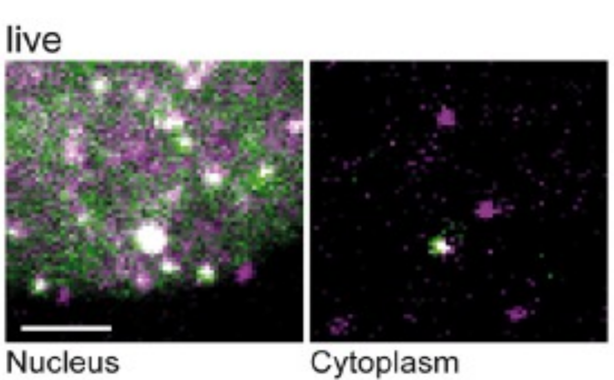
TREAT: 3'-RNA end accumulation during turnover

Single-mRNA imaging of RNA degradation in single cells

reporter RNA

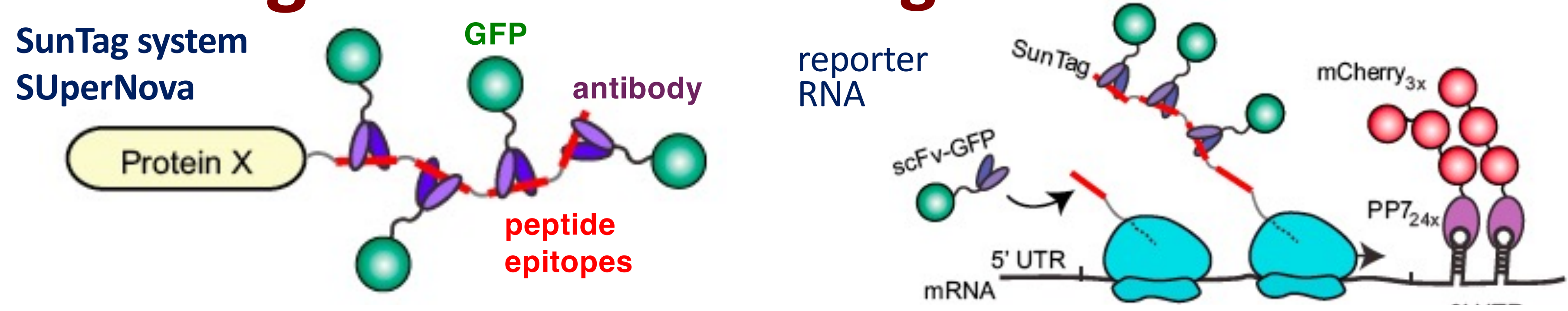






Horvathova et al, Mol Cell, 2017

SunTag: translation of single mRNAs *in vivo*



Translation visualized by labeling nascent peptide epitopes with antibody-GFP

Multivalent fluorescent amplification of nascent peptide signal

mRNA

Translational stress response

Polysome signal

Time

Local translation and active transport of polysomes

Polysome mobility analysis

Polysome tracing

Translation dynamics over time

GFP fluorescence

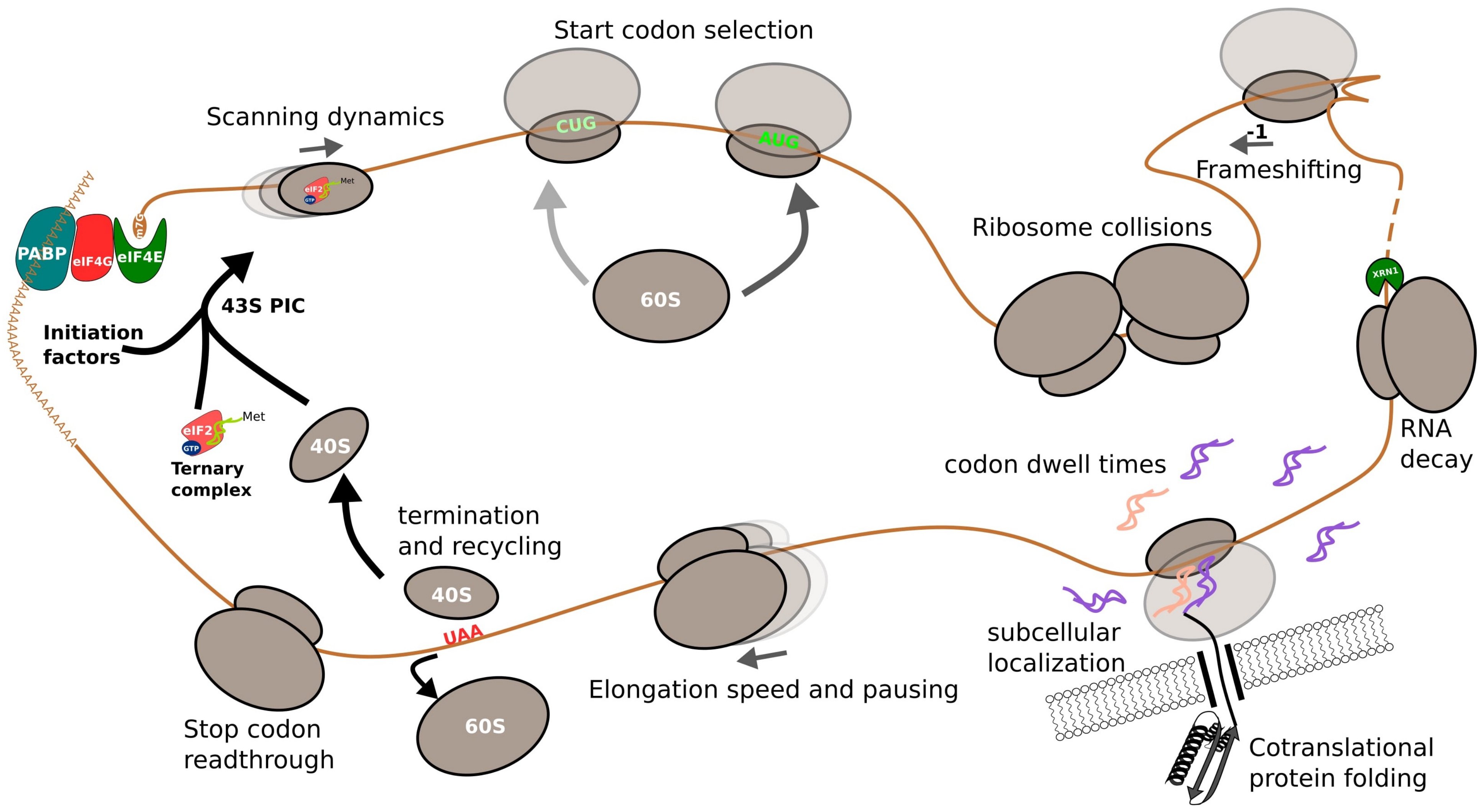
a b c d

Time

mCherry-labeled mRNA tethered to the membrane

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

Translatome analyses



Michał Świrski, 2024

Translatome analyses

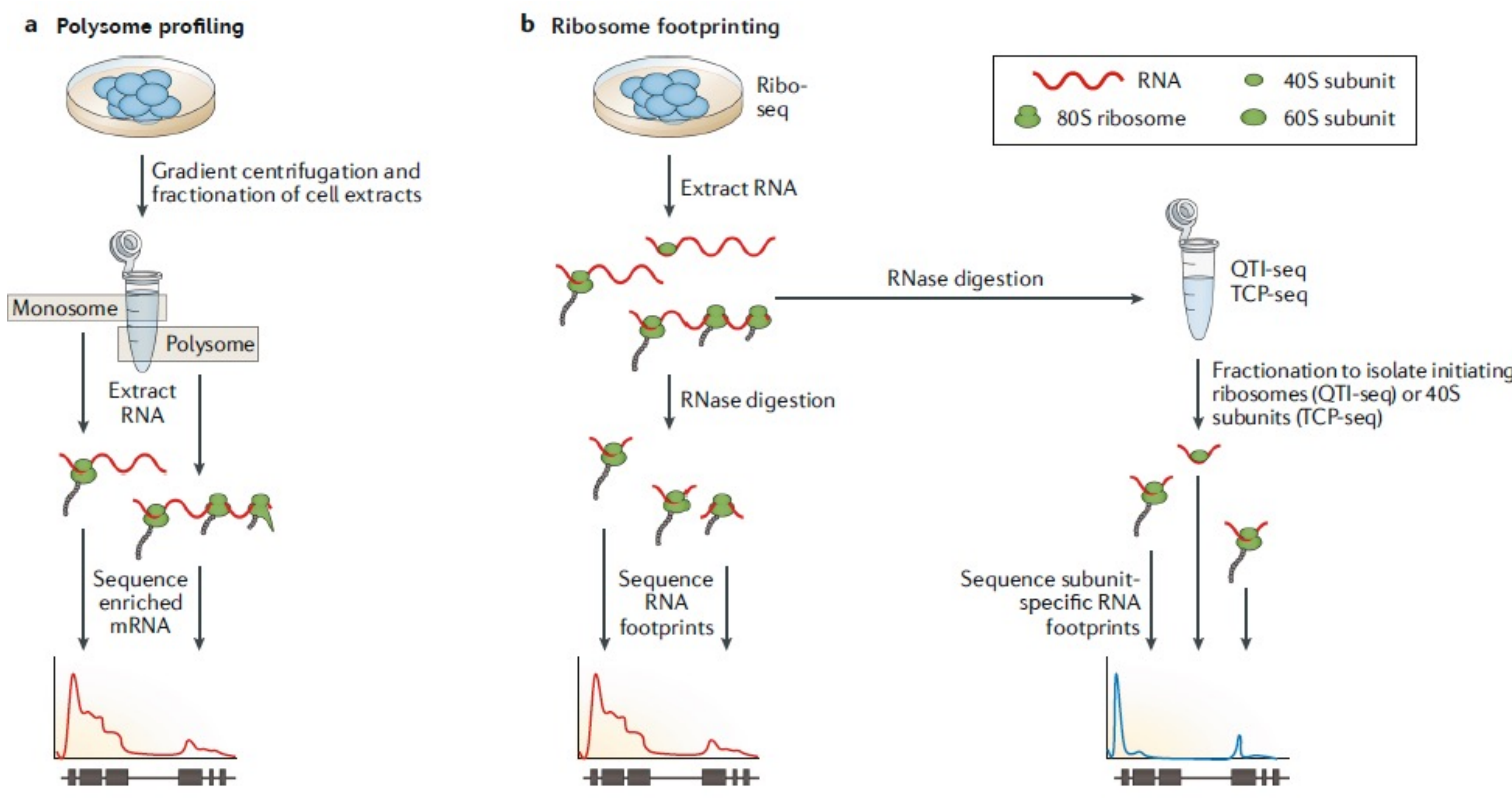
Ribosome Profiling
Ribosome Mapping
Ribo-seq

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

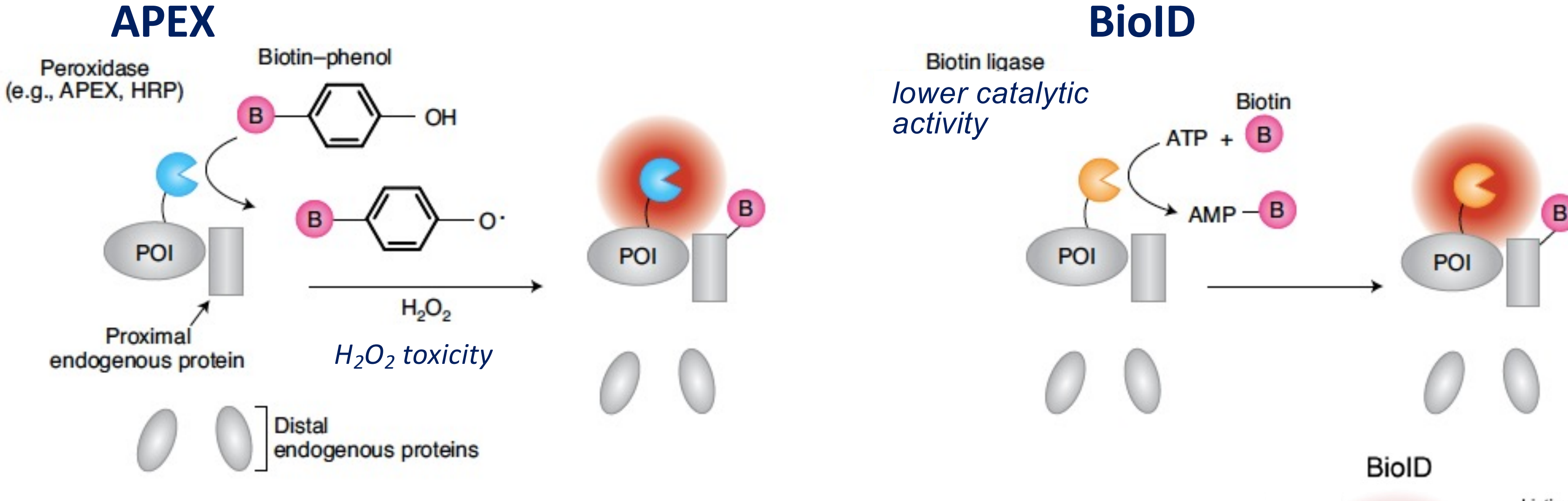
Translatome analyses

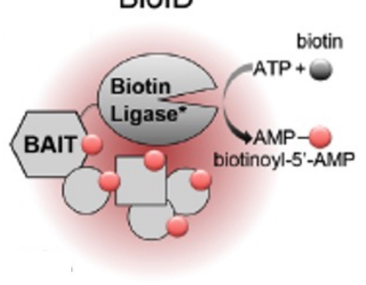


Stark et al, Nat Rev Genet, 2019

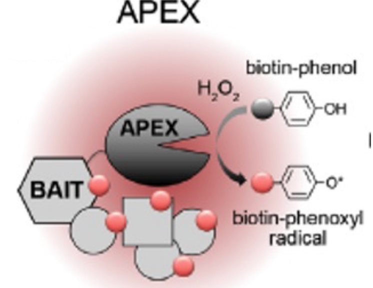
APEX and BioID

Proximity biotin-based labeling methods



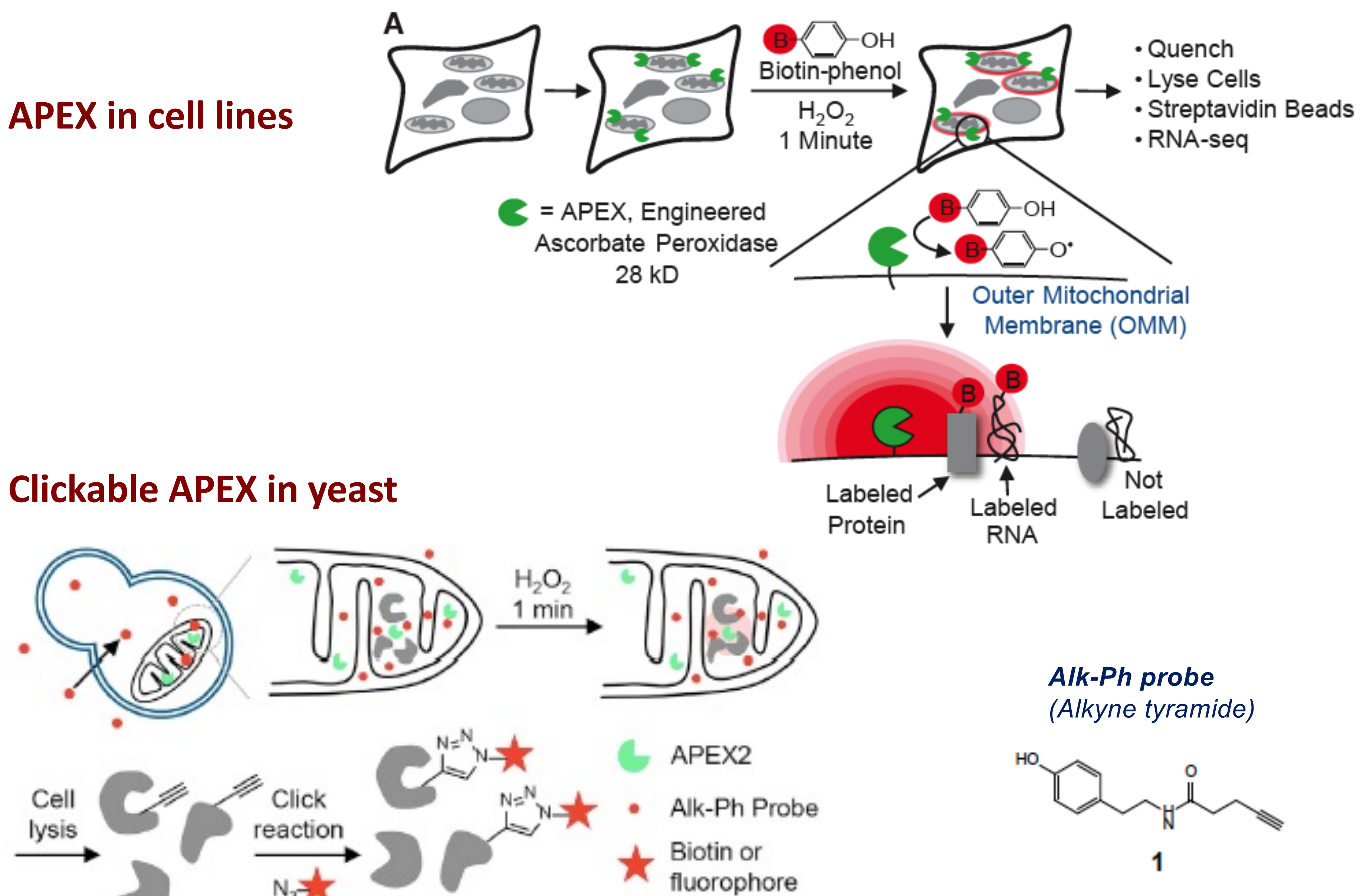


- An enzyme (**BioID**- biotin ligase; **APEX-** engineered ascorbate peroxidase) is targeted to a specific subcellular location (e.g. mitochondria) via fusion with protein markers or signal peptides.
- A probe (biotin or biotin-phenol) is enzymatically converted to biotin-conjugated reactive intermediates that react with nearby proteins/RNAs.
- Biotinylated proteins or RNAs are enriched with streptavidin beads and identified by mass spectrometry or RNA-seq, respectively.



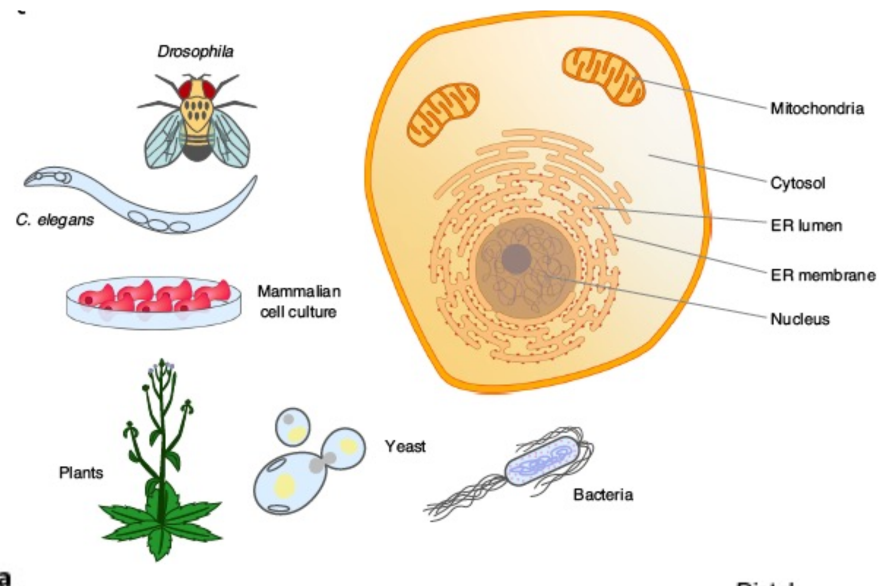
Qin et al, NatMethods, 2021; Li et al, Cell Chem Biol , 2020

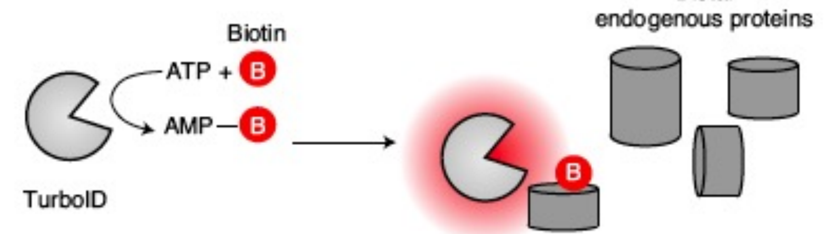
APEX

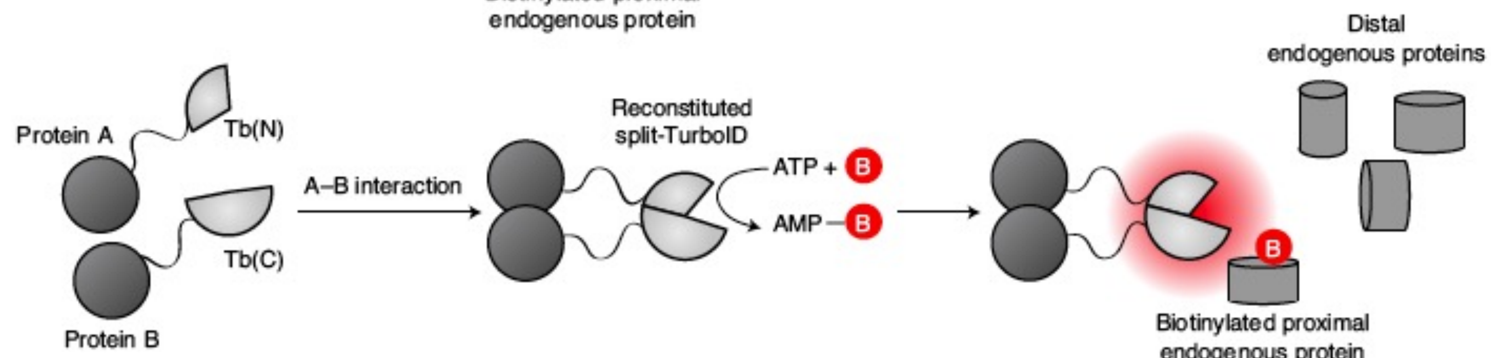


Fazal et al, Cell, 2019; Trinkle-Mulcahy, F1000Research, 2020; Qin et al, NatMethods, 2021; Li et al, Cell Chem Biol , 2020

TurboID







TurboID

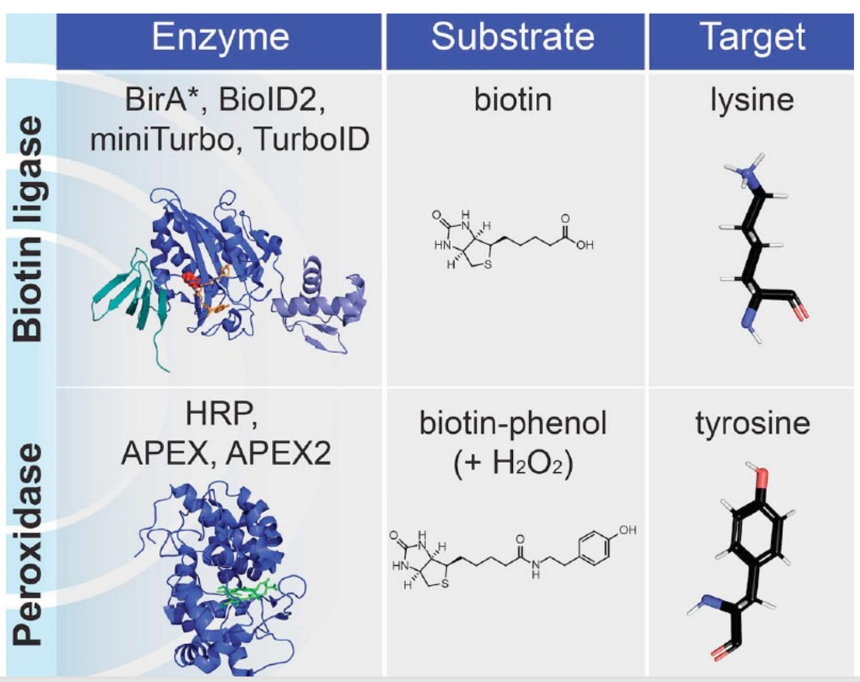
- Engineered, high activity biotin ligase.
- Uses ATP to convert biotin into biotin-AMP that covalently labels proximal proteins.
- Non toxic system.
- Gives higher temporal resolution and broader application *in vivo.*

Split-TurboID

- Consists of two inactive fragments of TurboID that are brought by protein-protein or organelle-organelle interactions.
- Has better targeting specificity than full-length enzymes.

Cho et al, Nat Protocols 2020

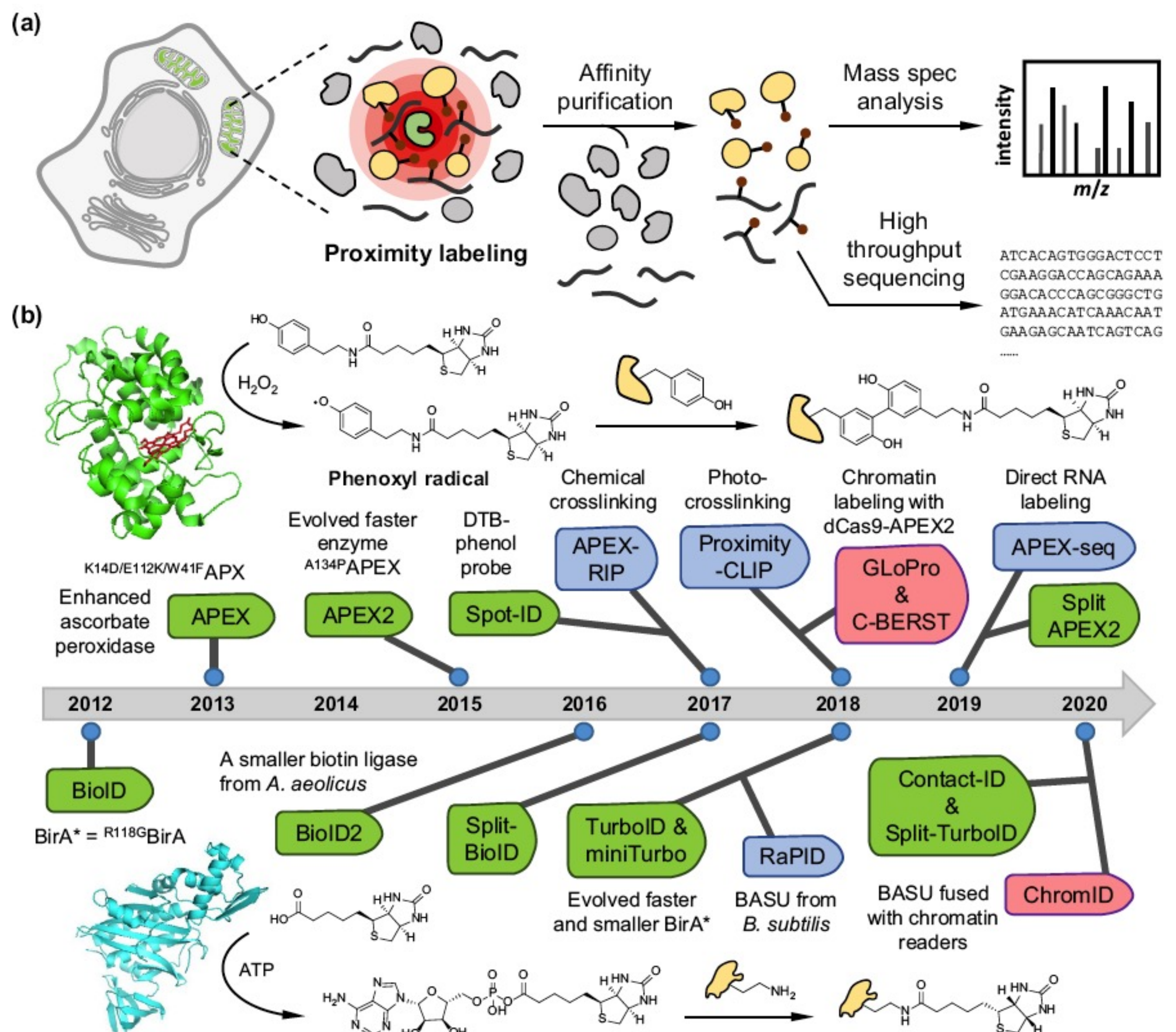
Proximity labeling systems



Enzyme	Type	Size (kDa)	Labeling time	Modification sites	Advantages	Limitations
APEX	Peroxidase	28	1 min	Tyr, Trp, Cys, His	High temporal resolution; versatility for both protein and RNA labeling	Limited application in vivo because of the toxicity of H_2O_2
APEX2	Peroxidase	28	1 min	Tyr, Trp, Cys, His	High temporal resolution; versatility for both protein and RNA labeling	Limited application in vivo because of the toxicity of H_2O_2
HRP	Peroxidase	44	1 min	Tyr, Trp, Cys, His	High temporal resolution; versatility for both protein and RNA labeling	Limited application in vivo because of the toxicity of H_2O_2; limited to secretory pathway and extracellular applications
BioID	Biotin ligase	35	18 h	Lys	Non-toxic for in vivo applications	Poor temporal resolution as a result of low catalytic activity
BioID2	Biotin ligase	27	18 h	Lys	Non-toxic for in vivo applications	Poor temporal resolution as a result of low catalytic activity
BASU	Biotin ligase	29	18 h	Lys	Non-toxic for in vivo applications	Poor temporal resolution as a result of low catalytic activity
TurboID	Biotin ligase	35	10 min	Lys	Highest activity biotin ligase; non-toxic for in vivo applications	Potentially less control of labeling window as a result of high biotin affinity
miniTurbo	Biotin ligase	28	10 min	Lys	High activity; non-toxic for in vivo applications; smaller than TurboID	Lower catalytic activity and stability as compared to TurboID

Qin et al, Nature Methods, 2021

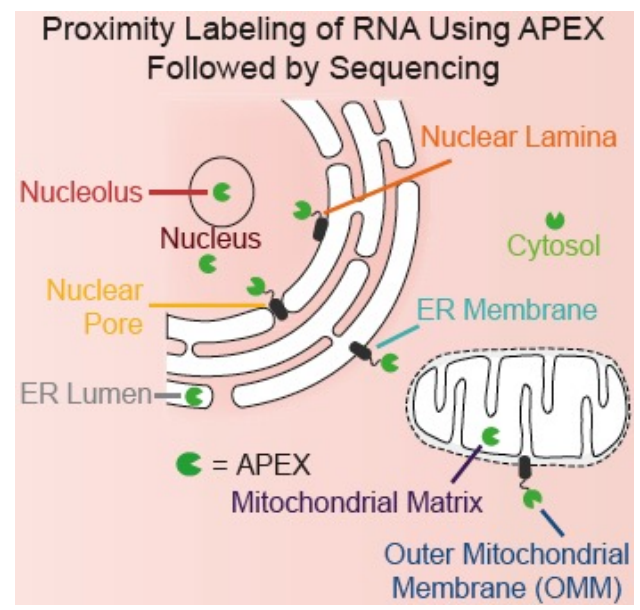
APEX



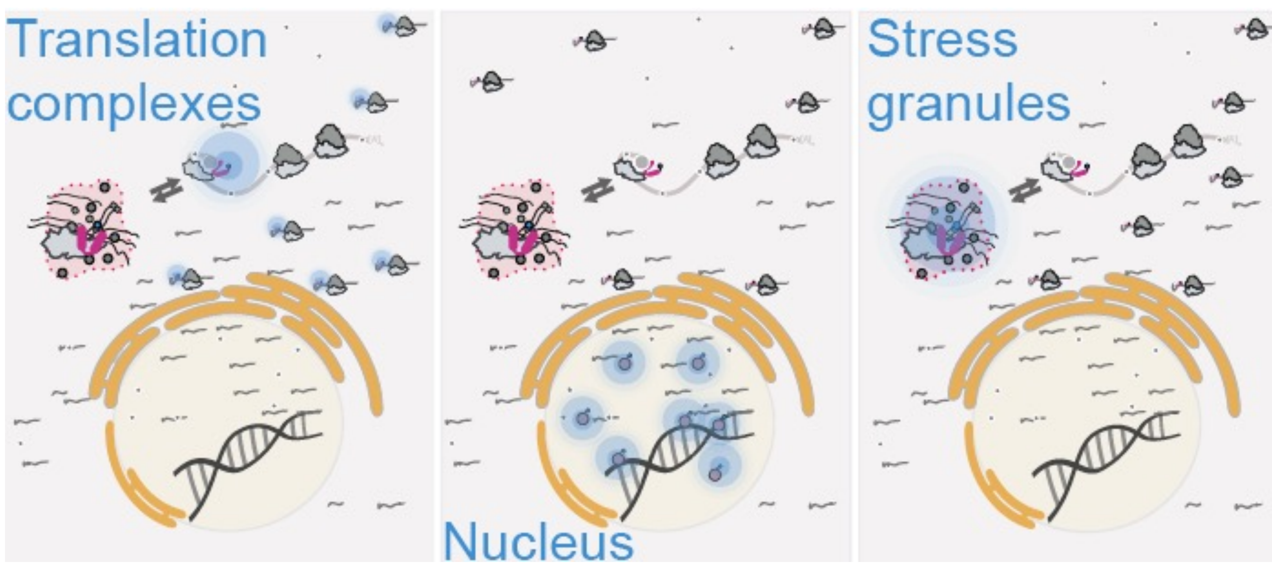
Zhou and Zou Curr Op Chem Biol 2020

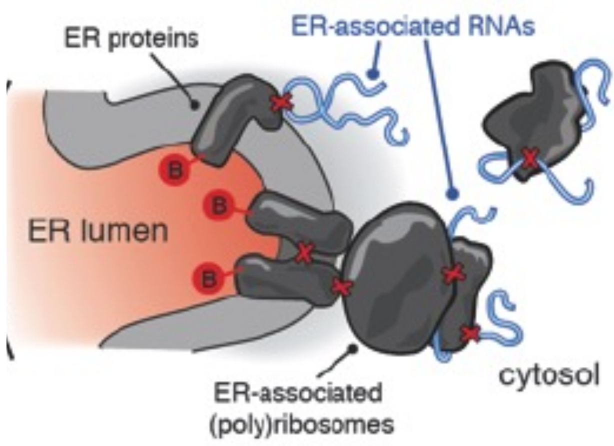
APEX: applications

Protein and RNA localization



RNP organization - translation, granule composition

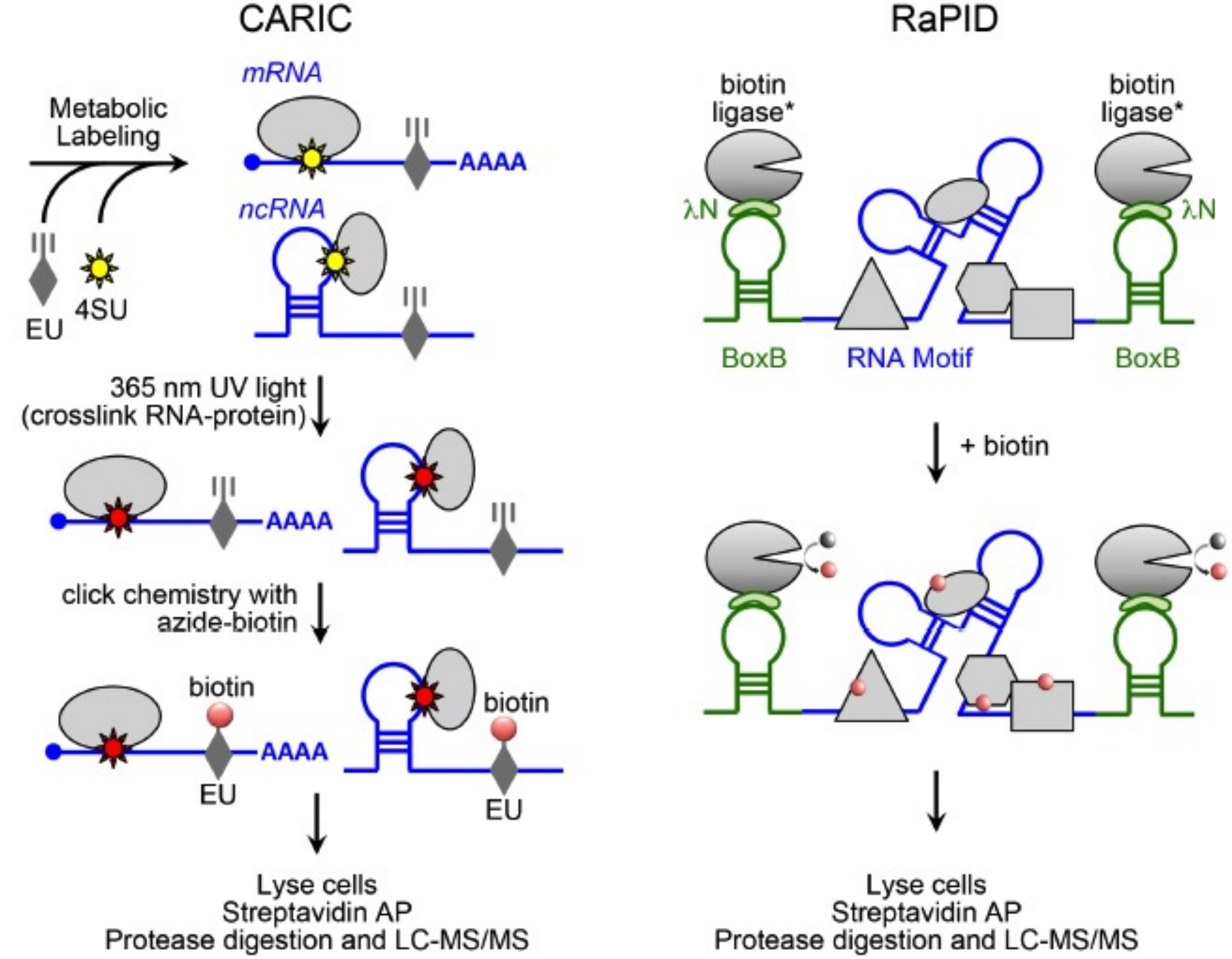




Fazal et al, Cell, 2019; Padron et al, Mol Cell, 2019

APEX: applications

Protein-RNA (RNP) complexes



Trinkle-Mulcahy, F1000Research, 2020

SUMMARY or HOW TO PASS THE EXAM?

- THEORY

- METHODS

- GENERAL IDEAS, CONCEPTS, SOLUTIONS

- PATHWAYS

- MECHANISMS