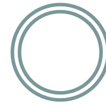
Ecole-Chercheur 2017

De l'expression des gènes aux réseaux

Module 1 : Les protocoles de sequencage a haut debit des ARNs et leurs applications

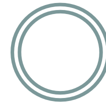
Jérémie Bazin (PostDoc IPS2)

Outline



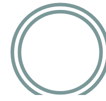
- Global transcriptome analysis : Goals, technical approaches and potential bias
- How to capture RNA subpopulations with RNA-sequencing : Concepts and Protocols
- Address biological questions and uncover molecular mechanisms with RNA-seq
- Combine technologies to access multiple gene expression regulations tiers in specific cells

RNA-Seq or Transcriptome Sequencing

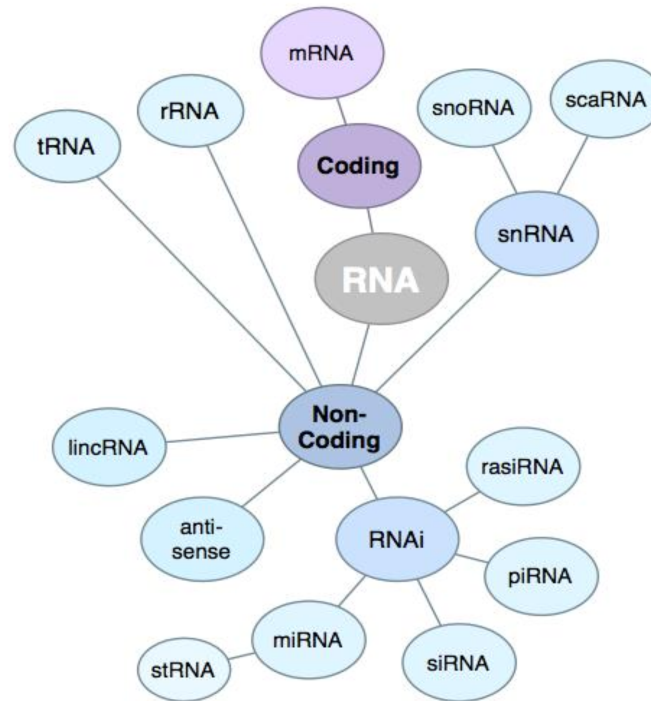


- **Transcriptome**
- It is a set of all transcripts in any given cell, all products of the transcriptional machinery.
- It includes coding (protein encoding) and non-coding RNA (not protein encoding).

RNA-Seq or Transcriptome Sequencing

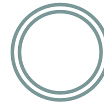


Transcriptome: RNA World



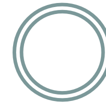
http://finchtalk.geospiza.com/2009/05/small-rnas-get-smaller.html

Goals to study the transcriptome :



- Biological question ?
  - What kind of RNA (mRNA, ncRNA, smallRNA, …) ?
- Quantification ?
  - Abundance of transcripts between different conditions
- Genome annotation ?
  - Identify genes, exons, splicing events, ncRNAs, etc.
  - Novel genes or transcripts

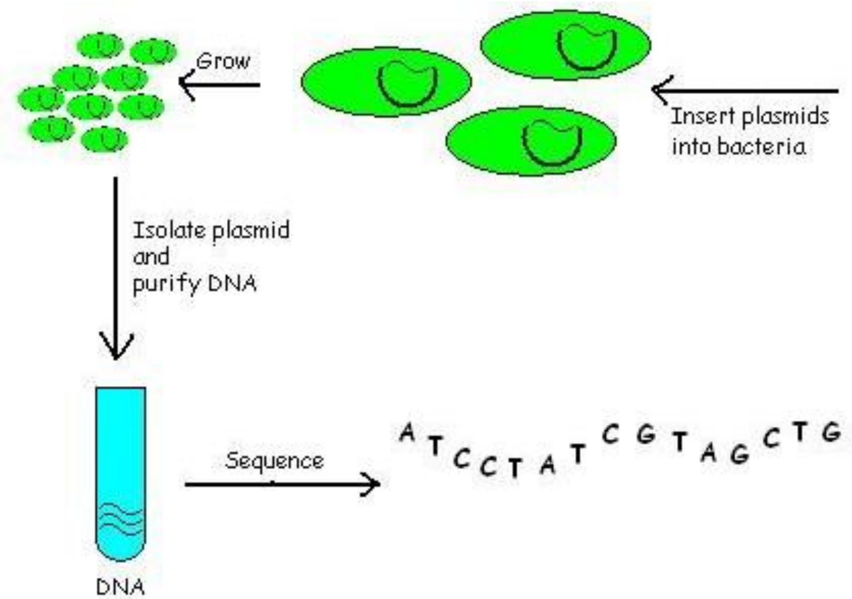
CAPTURE THE TRANSCRIPTOME : THE CDNA LIBRARY

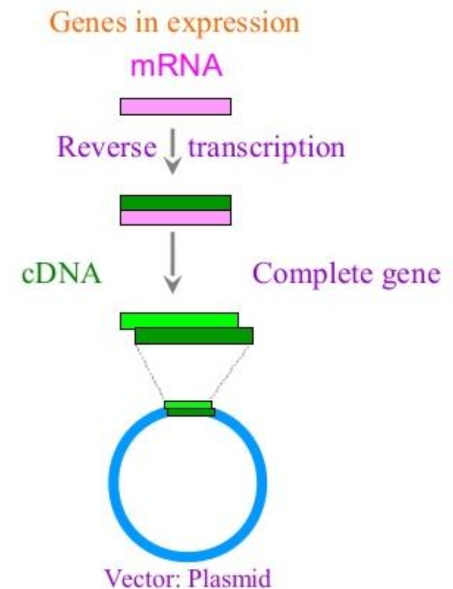


- cDNA, not RNA sequencing
- Old technique to study gene expression (Sim et al., Cell 1979)

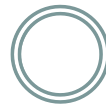
Cell, Vol. 18, 1303–1316, December 1979, Copyright © 1979 by MIT

Use of a cDNA Library for Studies on Evolution and Developmental Expression of the Chorion Multigene Families

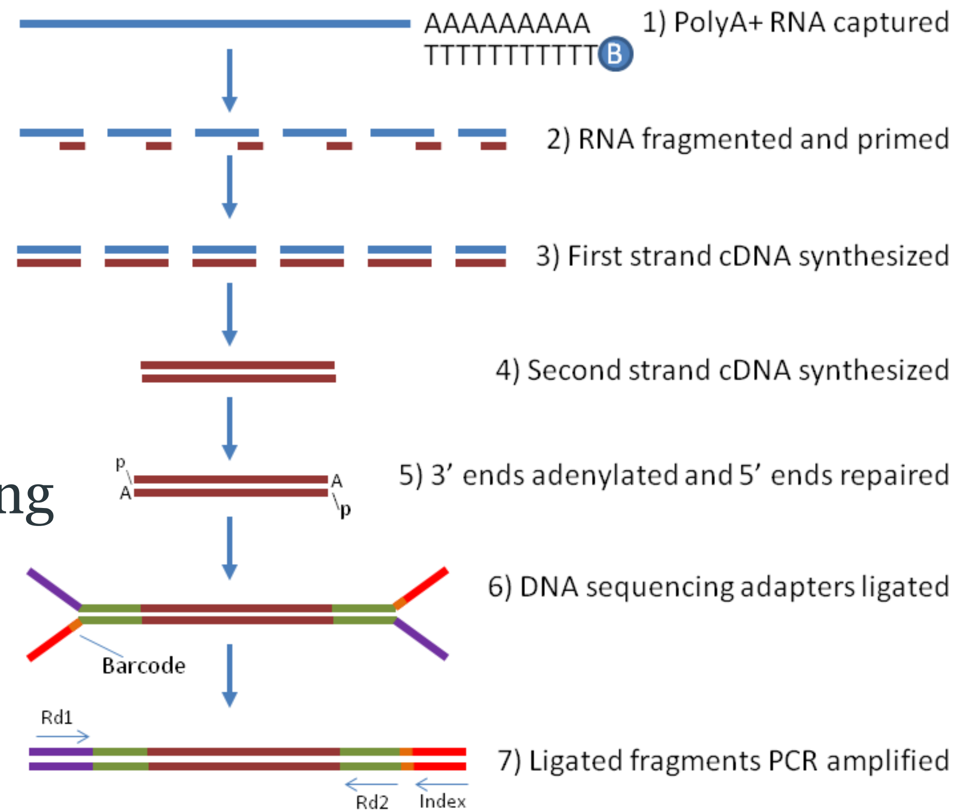




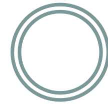
NEXT GENERATION CDNA LIBRARY



- Basic steps :
  - RNA Fragmentation
  - Reverse transcription
  - 5' /3' Adapter Ligation
  - PCR amplification
  - Library quantification
  - High throughput sequencing

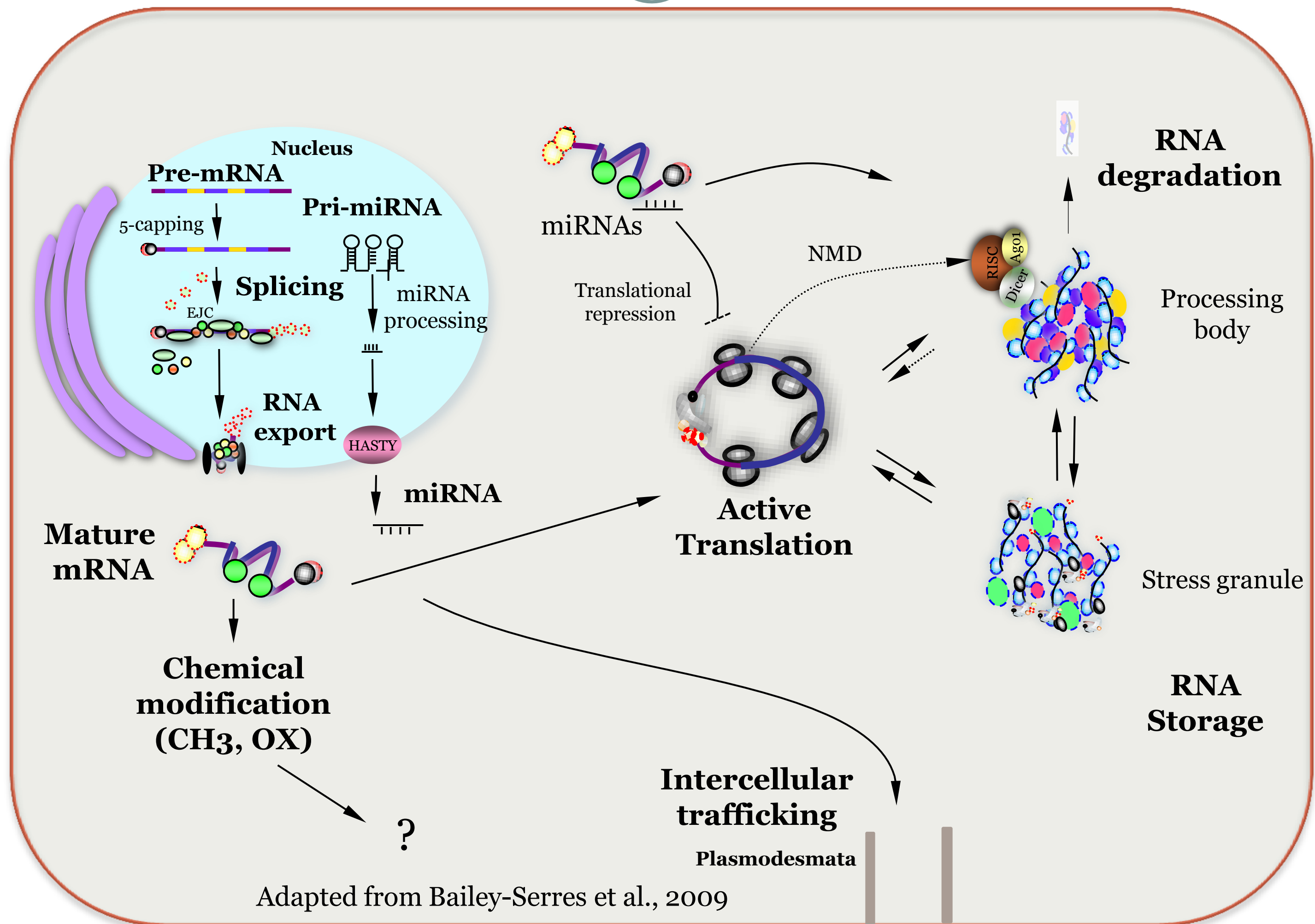


NEXT GENERATION cDNA LIBRARY

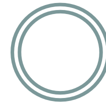


- Possible bias (most protocols)
  - RNA degradation during extraction
  - RNA fragmentation
  - PCR artifacts :
    - GC content sequences: depend on the DNA polymerase
    - PCR over-amplification : The number of cycle may need to be optimized : the less the better
  - Adapter dimer contamination

The life of an RNA, a journey of many pathways

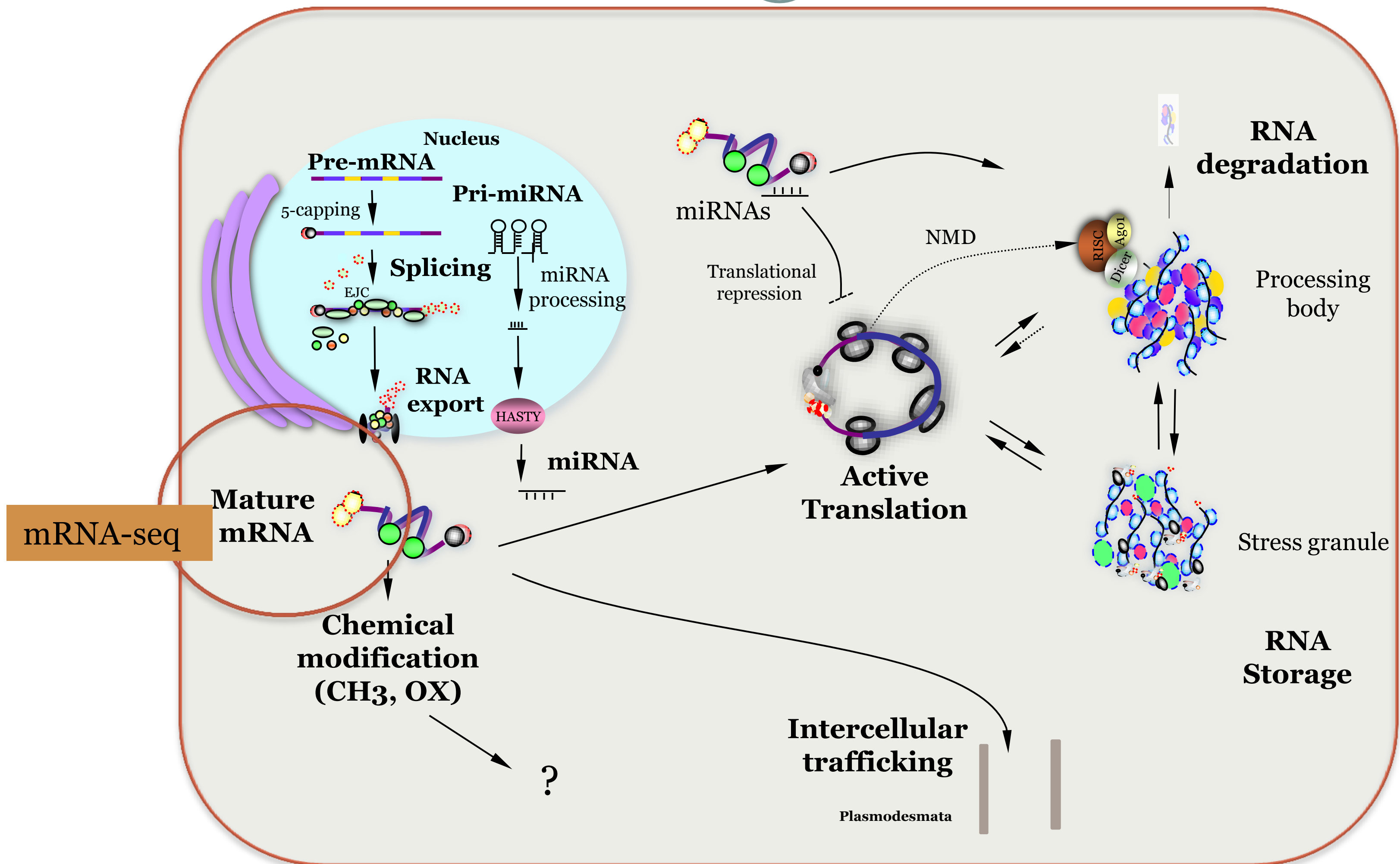


How to isolate RNA subpopulations

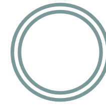


- Based on intrinsic chemical features of the RNA (Size, 5'/3' end)
- Subcellular localization (Nucleus, organelles…)
- Association with proteins complexes
- Combination of these approaches
- Everything is possible but need to be designed carefully !

Q: What is the effect of my favorite stress, mutation on nuclear gene expression ?

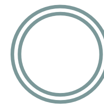


mRNA-seq

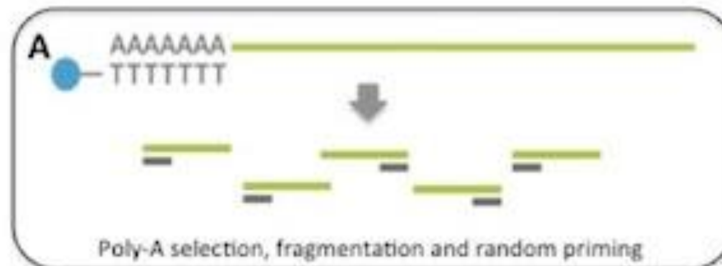


- mRNA features :
  - Broad size distribution,
    - Will require fragmentation before cloning
  - polyA tail, 5' Cap
    - PolyA tail can be used to enrich for mRNA among other RNA

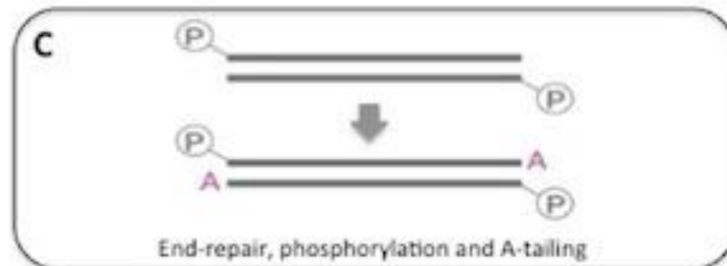
mRNA-seq



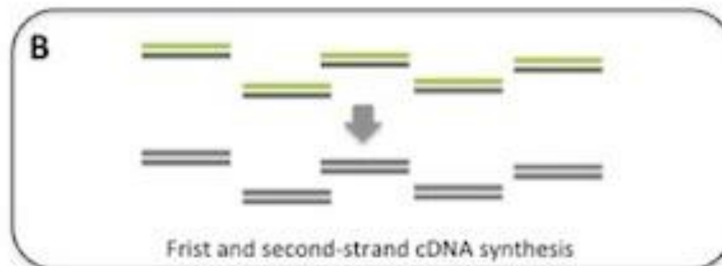
Illumina Tru-Seq RNA-seq protocol



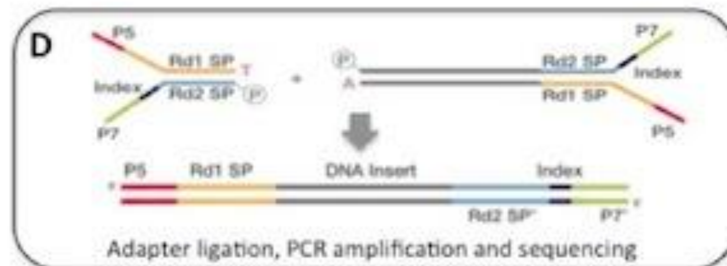
Poly-A selection, fragmentation and random priming



End-repair, phosphorylation and A-tailing



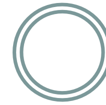
Frist and second-strand cDNA synthesis



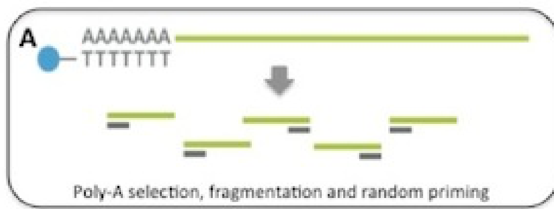
Adapter ligation, PCR amplification and sequencing

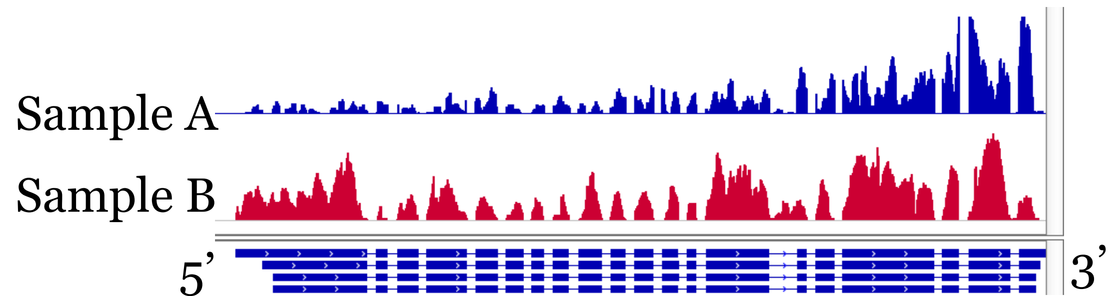
Library prep begins from 100ng-1ug of Total RNA which is poly-A selected (A) with magnetic beads. Double-stranded cDNA (B) is phosphorylated and A-tailed (C) ready for adapter ligation. The library is PCR amplified (D) ready for clustering and sequencing.

mRNA-seq

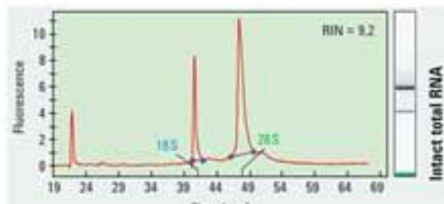


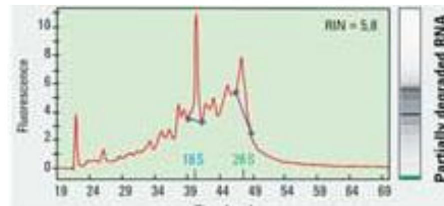
- Potential bias I
  - Based on purification of polyA RNA : sensitive to RNA degradation !!
  - Can create a 3'end biased coverage which may compromise quantification



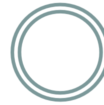


- Solutions : Check RNA integrity
  - Agilent Bioanalyzer (RIN>8)

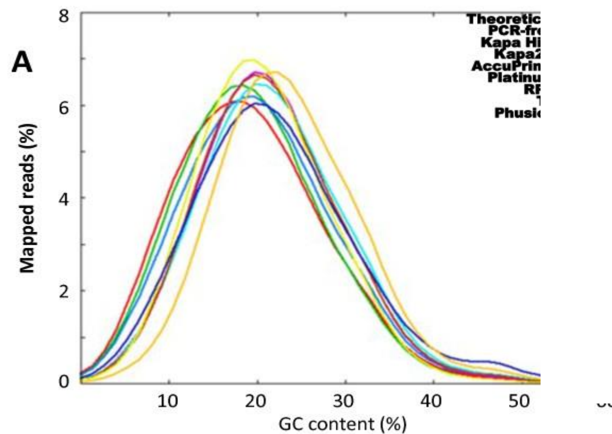


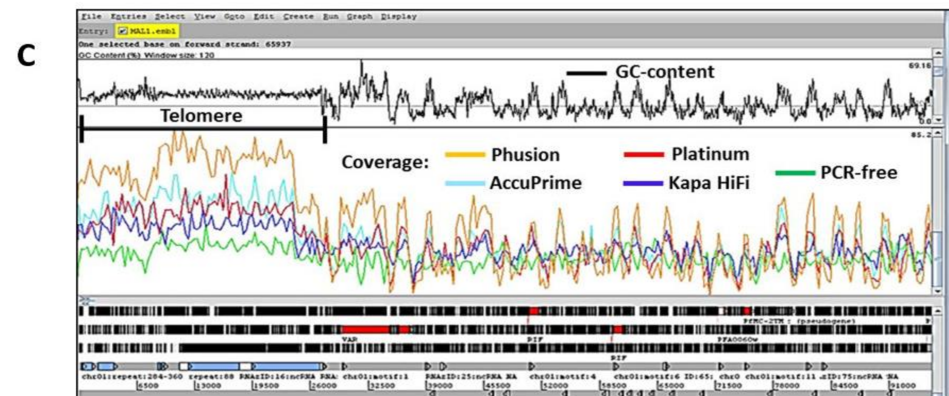


mRNA-seq



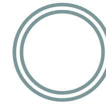
- Potential bias II
- PCR overamplification :
  - Reduce library complexity, results in overrepresented sequences.
- GC content
  - Polymerase dependent
  - Can be reduced by reducing PCR cycles



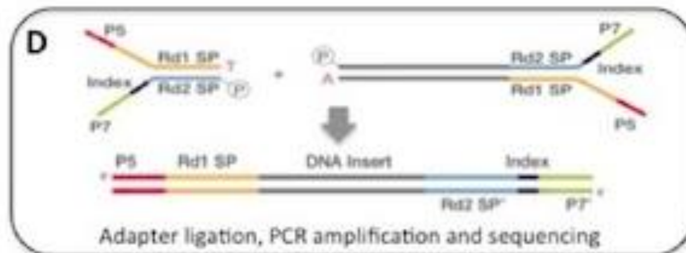


Oyola et al., 2012 BMC Genomics

mRNA-SEQ

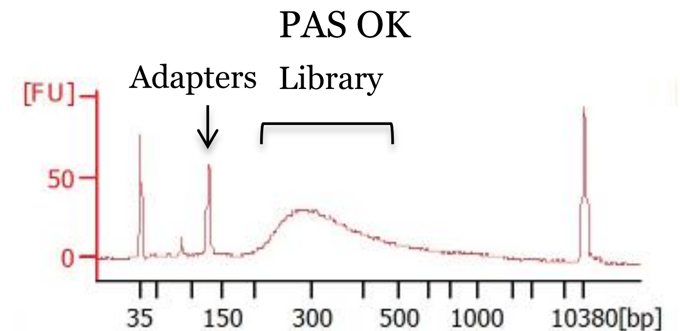


- Potential bias III
- Adapter dimer contamination:
  - Shorter fragment are sequenced preferentially = loss of usable reads

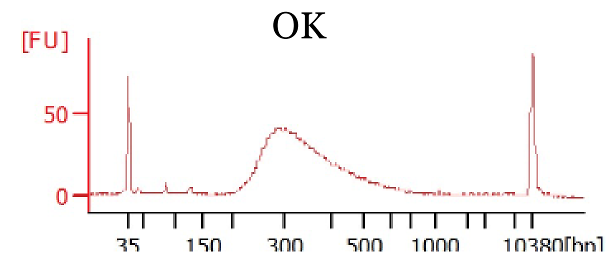


At this step, adapters can be ligated together.
Especially when RNA input is to low

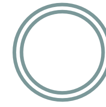
PAS OK



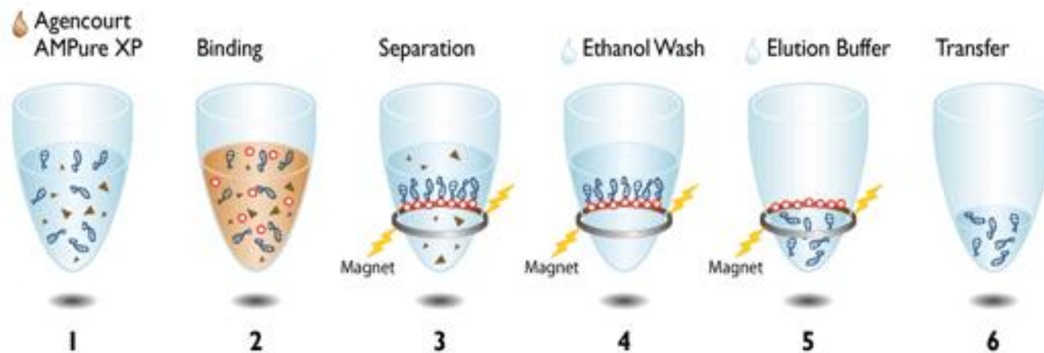
OK

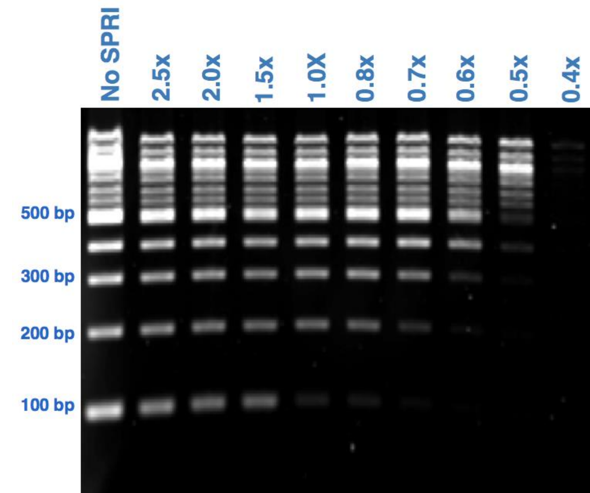


mRNA-seq

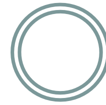


- AMPure XP bead purification
- At each step or the protocol, where buffer or removal of primer is required.
  - Is also used to separate dsDNA by size



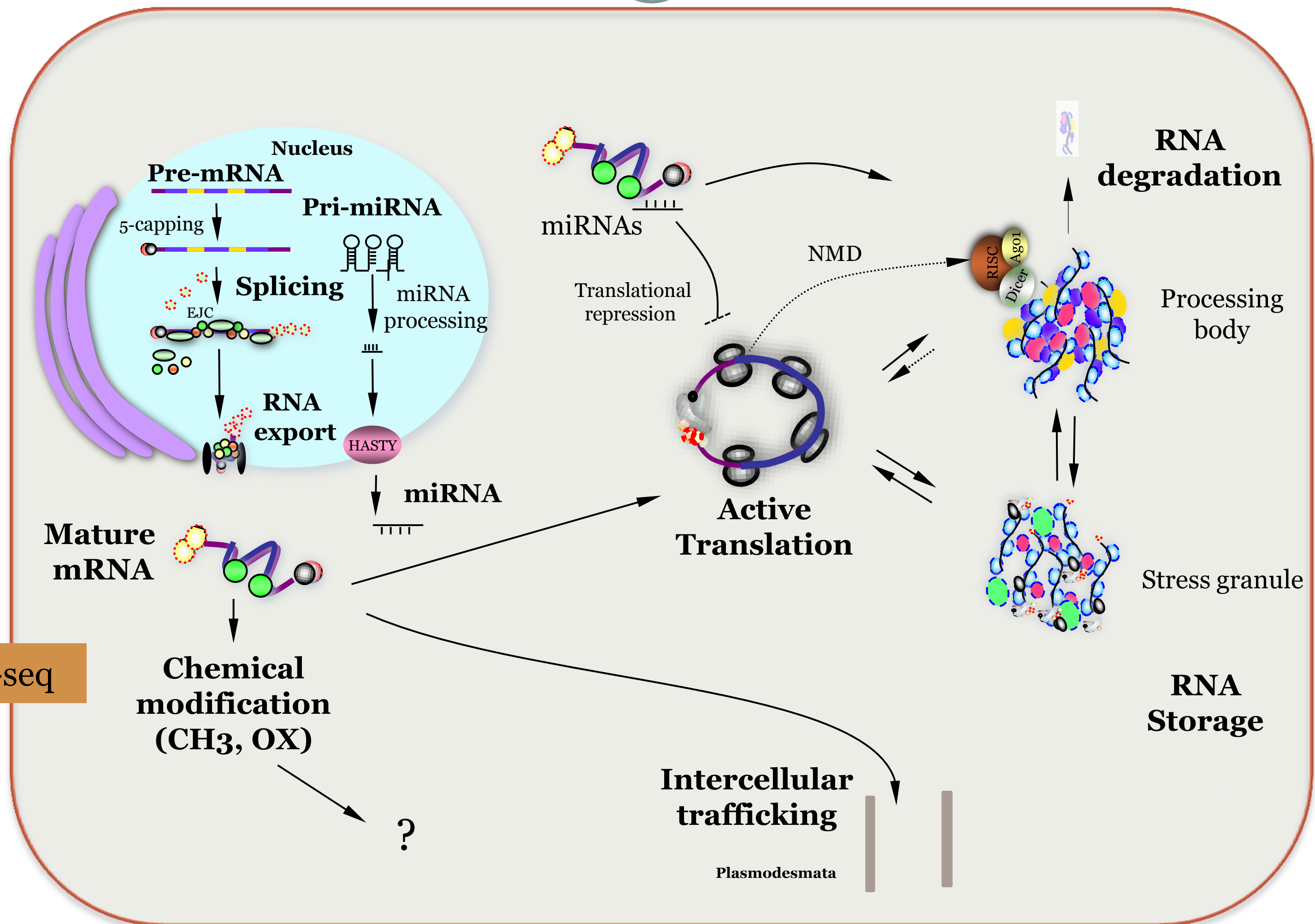


How to decrease the cost of mRNA-seq



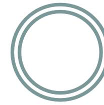
- Design your experiment carefully, use the right depth, more replicates is often better than more depth.
- Prices of sequencing are constantly decreasing but not the cost of library prep kits !
- You can use a home made protocol, this is just basic molecular biology!
  - Ex : Wang et al., Plos One 2011 : TrueSeq like protocol , 5$ per library vs 50$ for Illumina TrueSeq mRNA
- The amount of reagents Illumina TrueSeq and Script-seq protocols can be reduced to half (25$/Library)

Q: What is the effect of my favorite stress, mutation on global gene expression ?

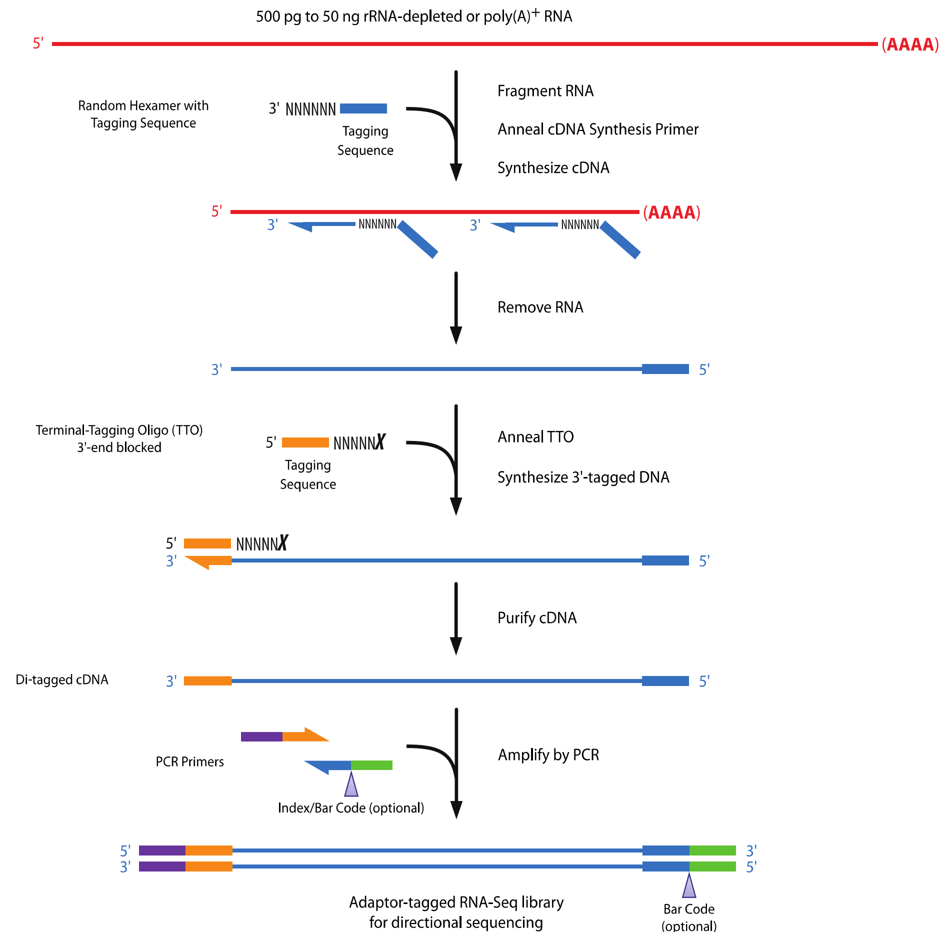


Total RNA-seq

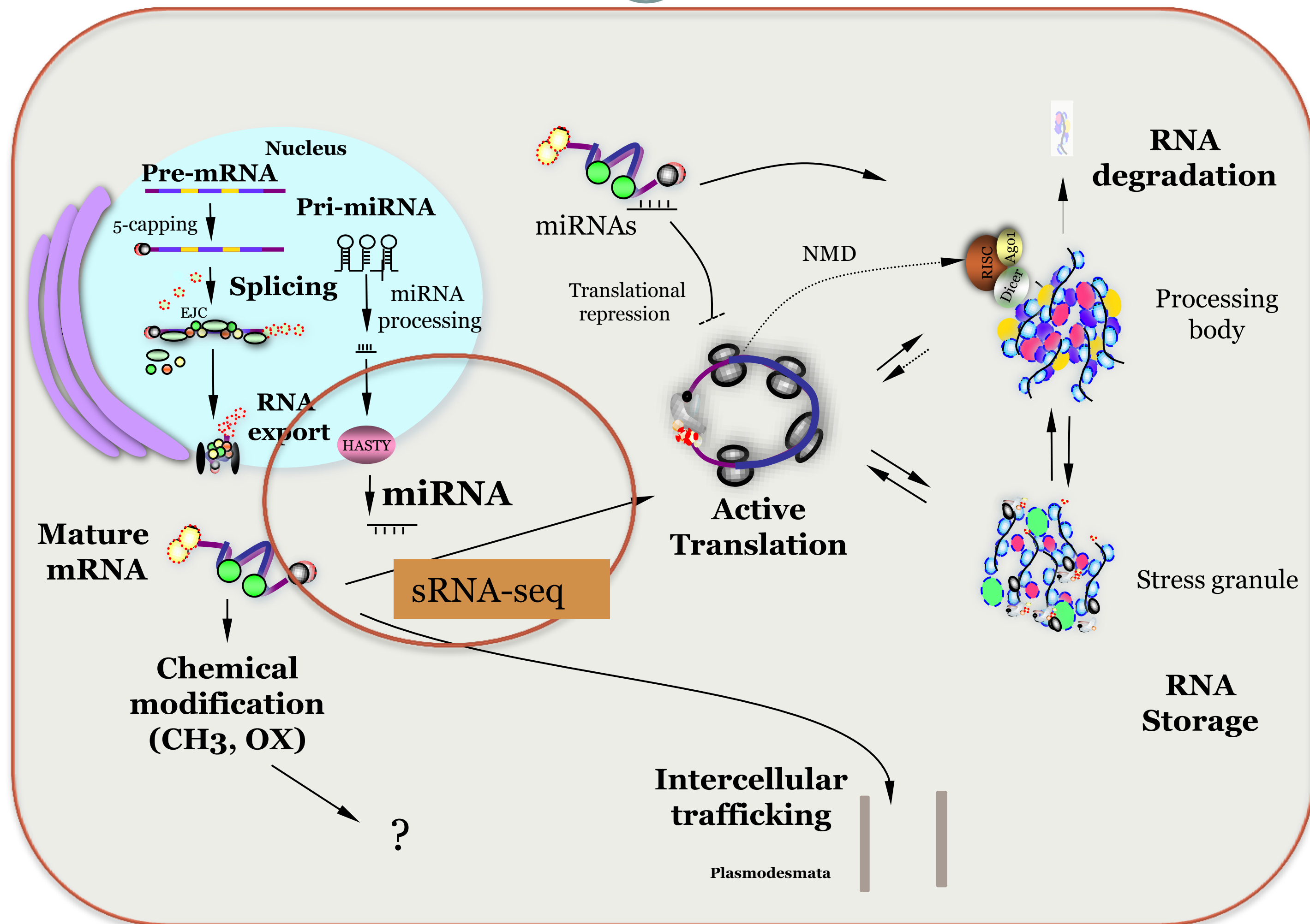
Total RNA seq



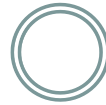
- Illumina Script-seq V2
  - Generally used after ribosomal RNA depletion (Ribo-zero kit)
  - Ligation of adapter on single stranded cDNA
  - Requires less RNA (50-500ng)
  - Less sensitive to RNA degradation
  - Sensitive to gDNA contamination
  - Much faster !



Q2: Which miRNAs are epressed in a particular condition/ Species

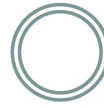


sRNA-seq

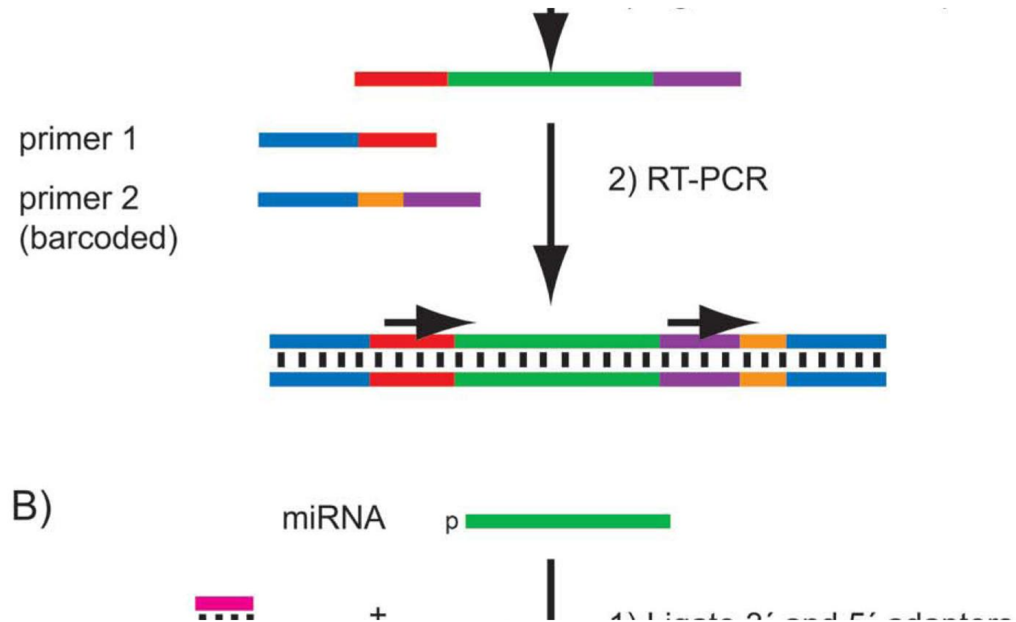


- sRNA features :
  - Narrow size distribution,
    - Do not require fragmentation, can be size selected
  - 5' phosphate end, 3'o-methylated (plant)
    - 5'P end can be used to ligate selectively sRNA

sRNA-seq



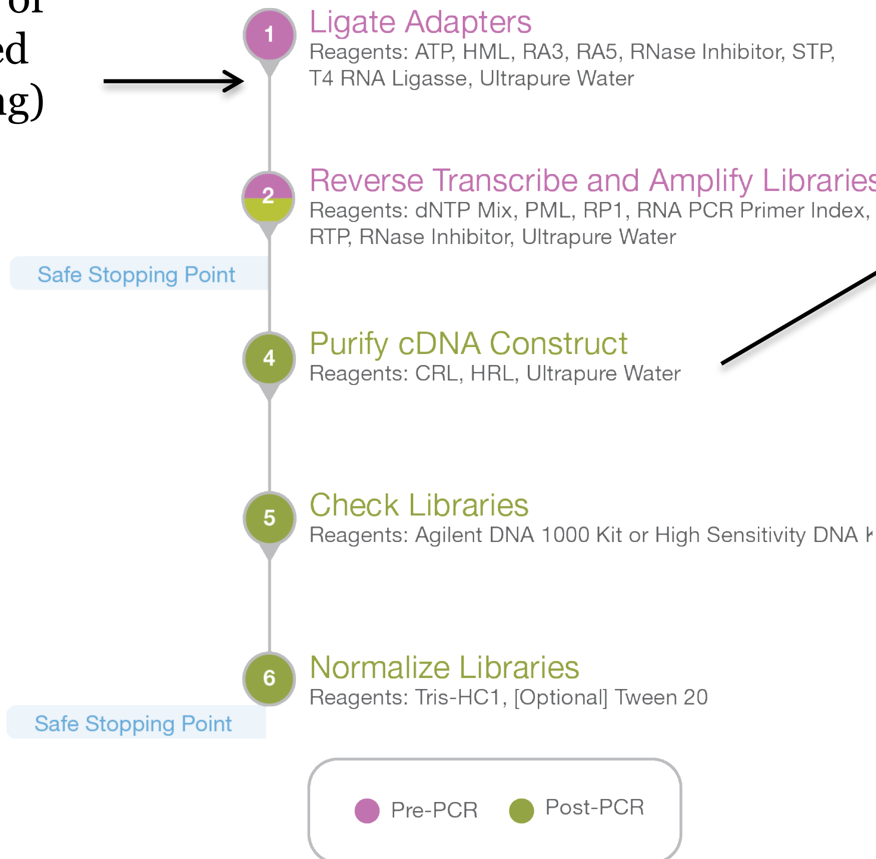
- Ligation of adapter directly on the end of the sRNA
- Uses the 5' phosphate end to select for miRNA
- Very sensitive to RNA degradation !
- Require a high amount of RNA.



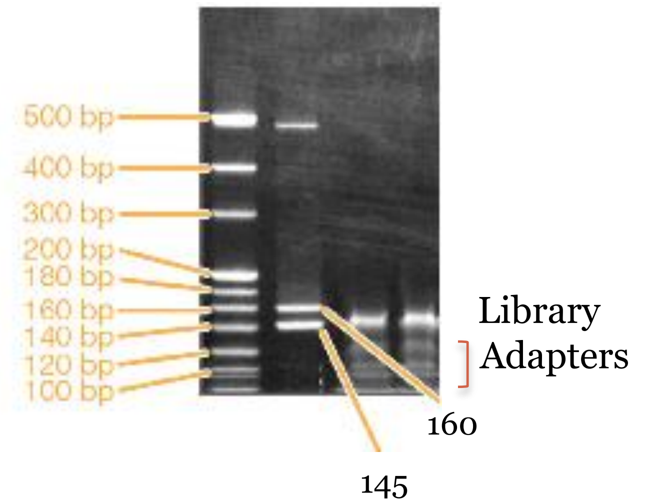
sRNA-seq

Illumina Small RNA Library Prep kit

Total RNA or size selected sRNA (50ng)



Separate library from contaminant on PAGE or Pippin prep



sRNA-SEQ

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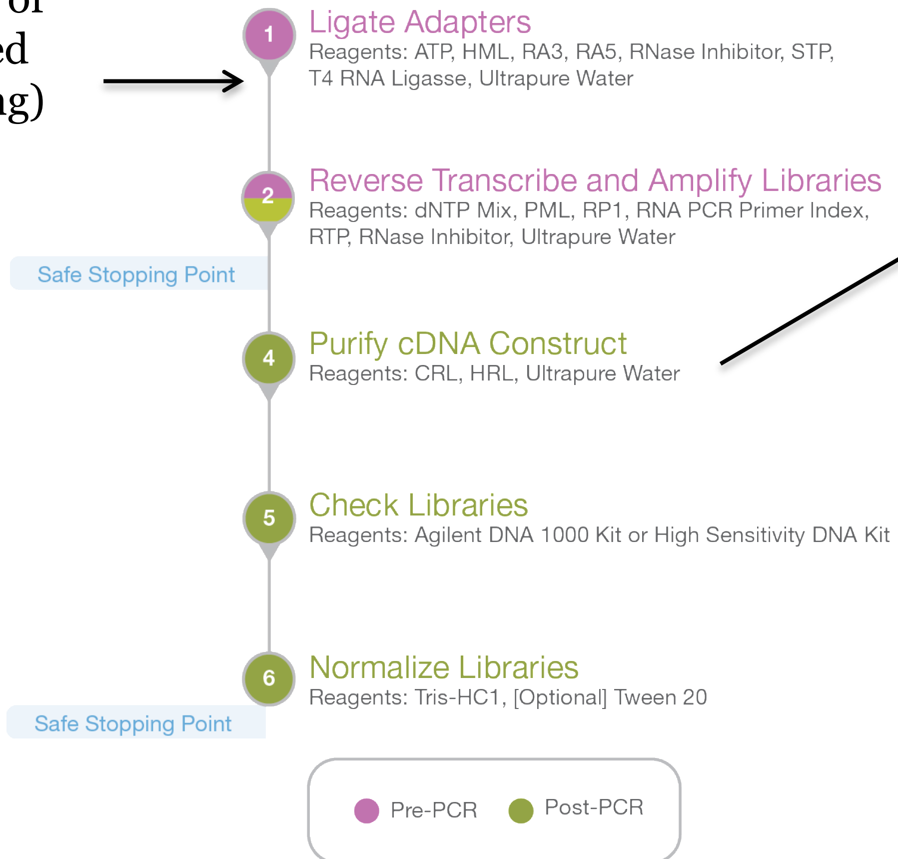
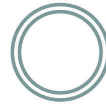
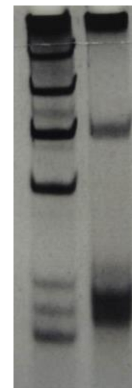


Separate library from contaminant on PAGE or Pippin prep

sRNA-seq

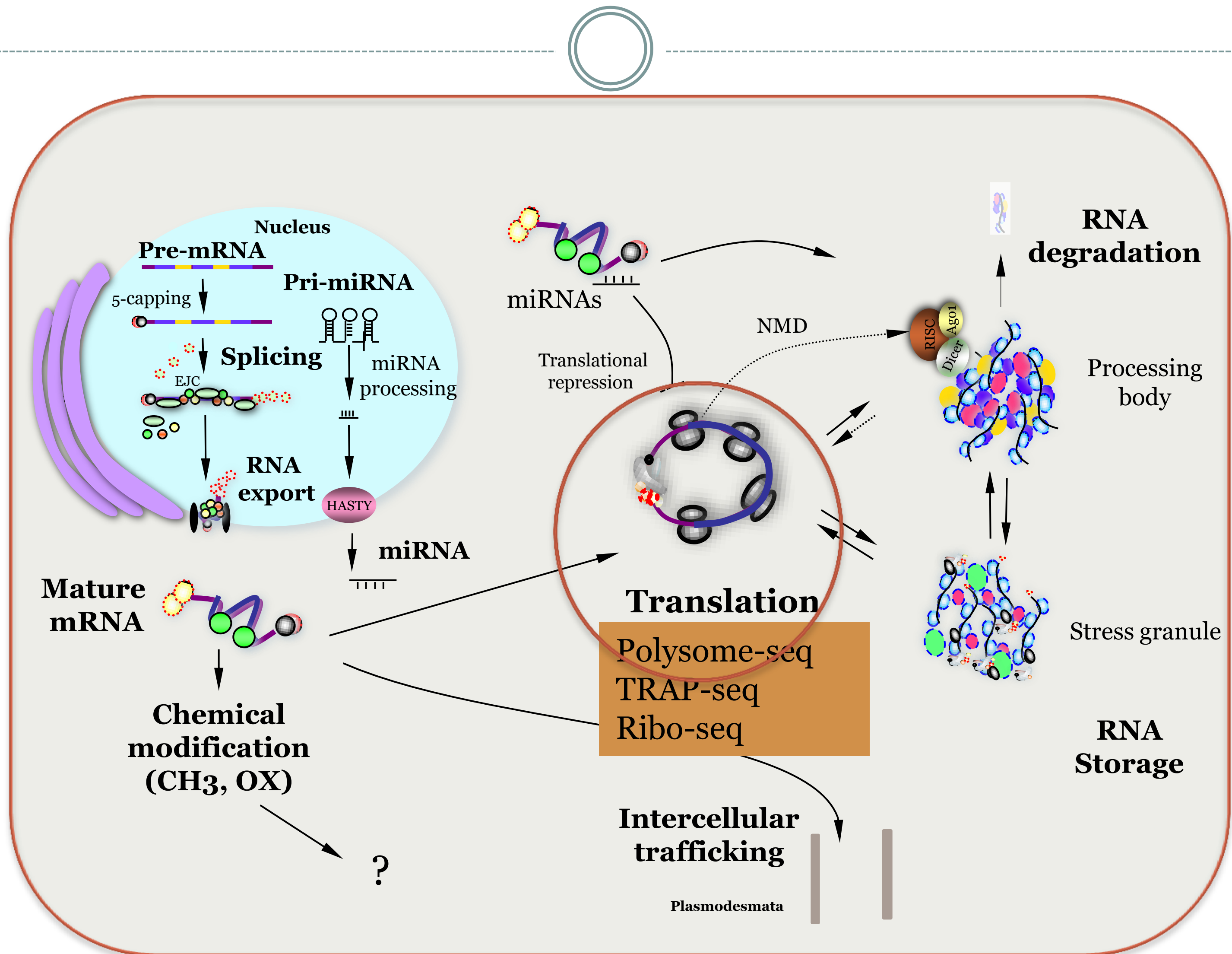


- A few recommendations:
  - Work with high integrity (RIN>=9) pure RNA
  - Extract with Trizol or miRNA kit (eg miRVana Ambion)
  - Start with a high amount of material (> 10µg)
  - Size select on a 15% denaturing UREA TBE PAGE gel to enrich for 18-25nt long RNA

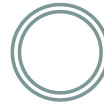


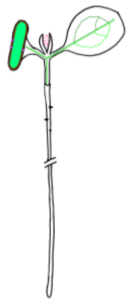
Small RNA

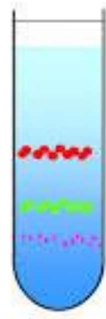
Q: Understand translation dynamics in response to an environmental stress

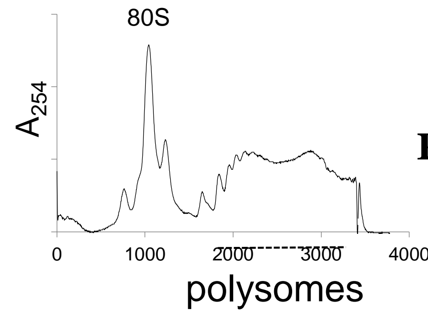


Capture actively translating mRNA

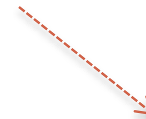




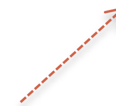


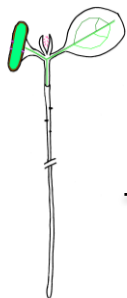


Polysomal RNA

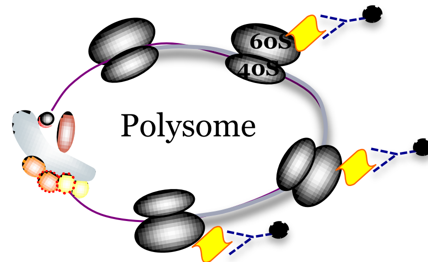


PolyA+ RNA-seq =Translatome



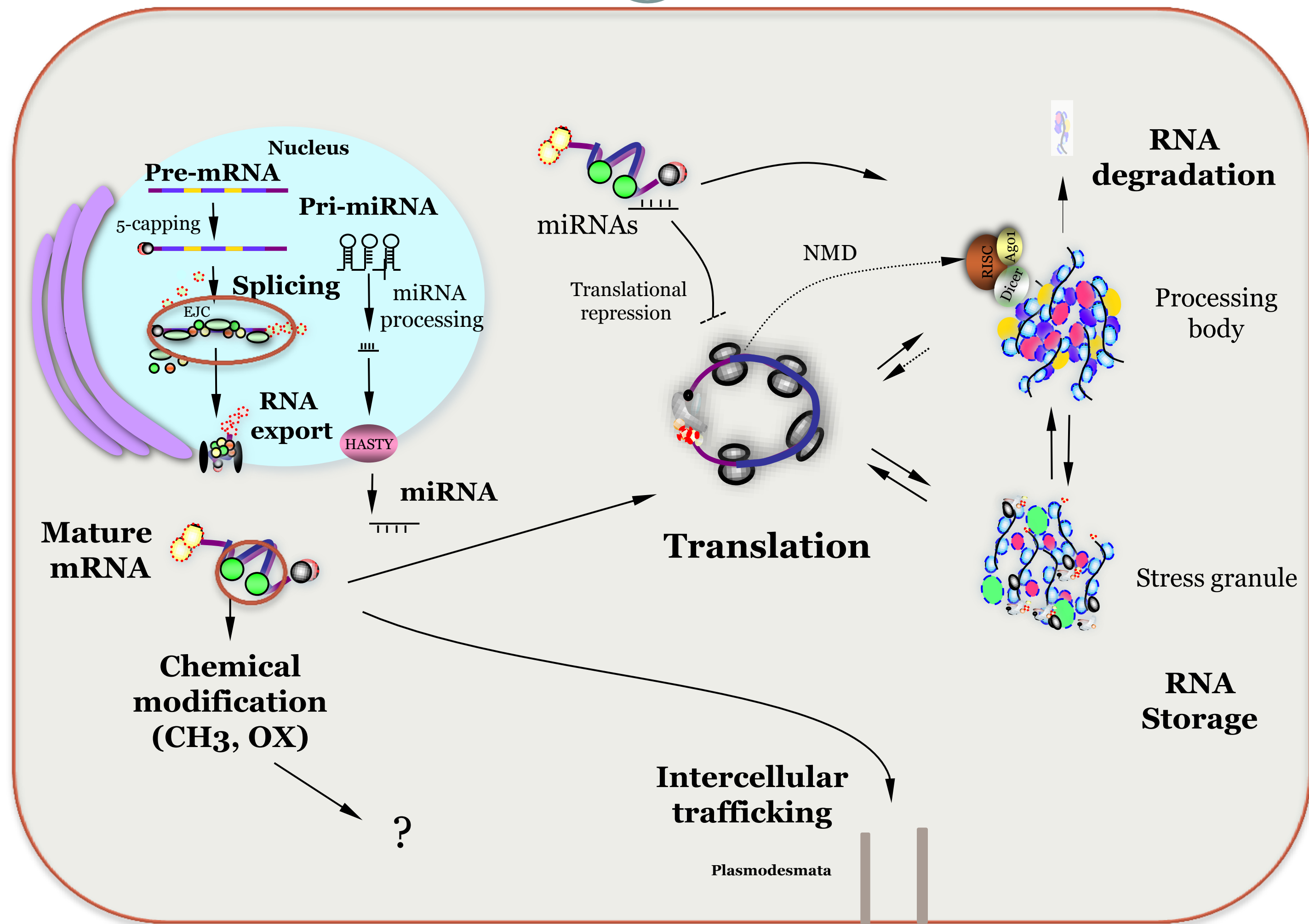


Translating Ribosome Affinity Purification (TRAP)



Polysomal RNA

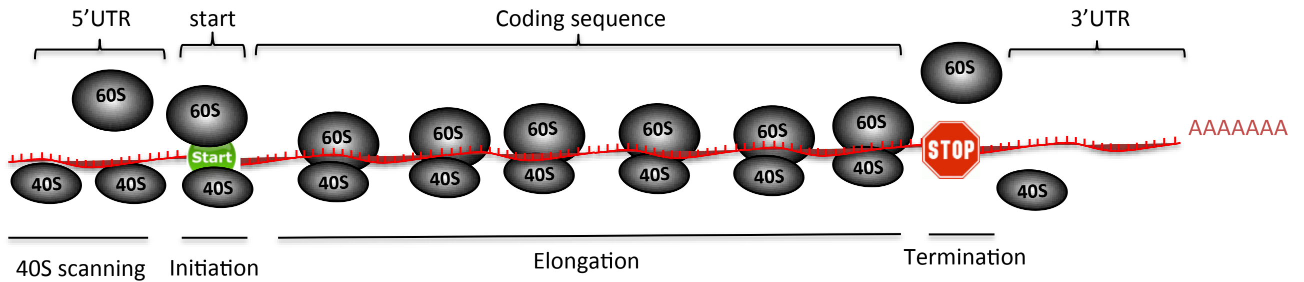
Q: Which RNA species are bound to an RNA binding protein ?



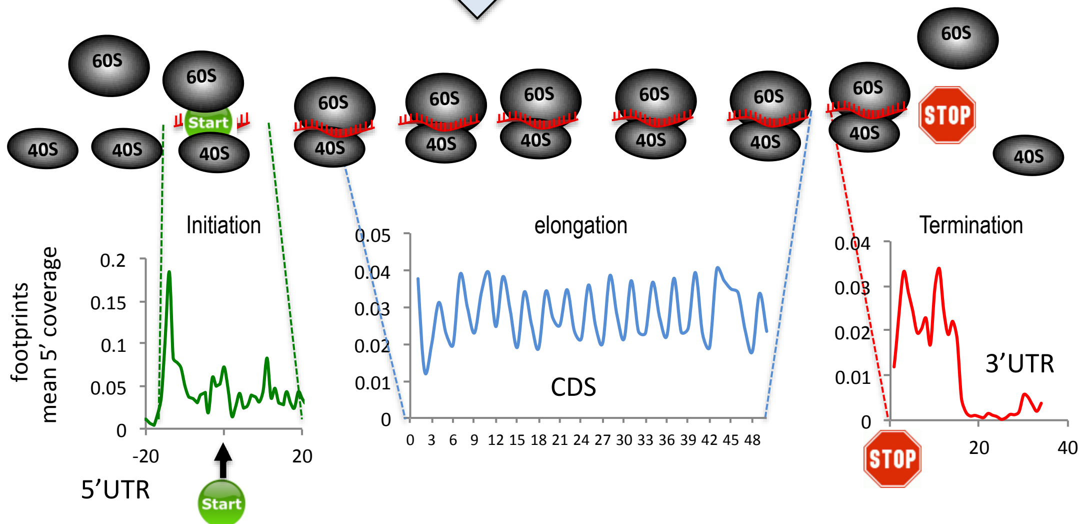
Jérémie Bazin 30/05/17

Q: What are the mechanisms of translational regulation ?

Ribo-Seq : Sequencing of ribosome footprints



1. RNAse1 digestion
2. Footprint library preparation
3. Sequencing /mapping to genome



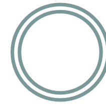
Coverage plots of all 5'UTR, CDS and 3'UTR

Arabidopis seedling roots

35S:FLAG-RPL18

Bazin et al., under review

Ribosome fooprint sequencing: Ribo-seq

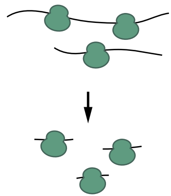


- Ribosome footprint features :
  - Size distribution : 26-32nt
    - Require optimal RNAse1 digestion
  - 5’ phosphate end,)
    - 5’P end can be used to ligate RNA adapter

Ribosome fooprint sequencing: Ribo-seq

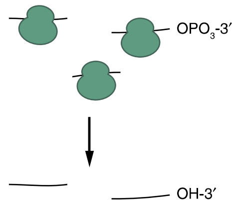
- Protocols :
  - Ingolia et al., 2012 Nat. Prot.
  - Illumina ArtSeq Kits

RNAse1 digestion

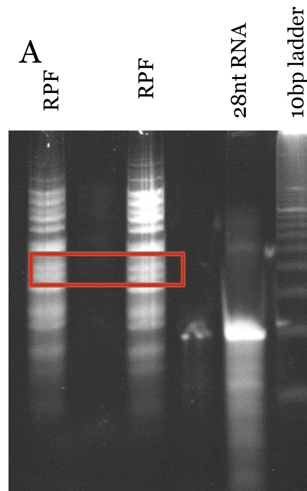


Steps 1–17: cell lysis, nuclease footprinting and ribosome recovery

Footprint purification

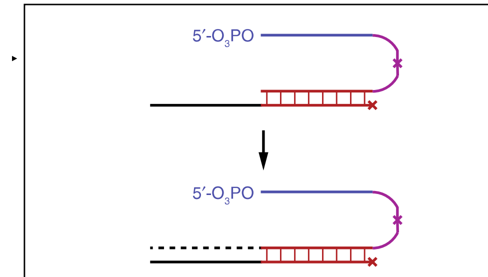


Steps 18–29: footprint fragment purification

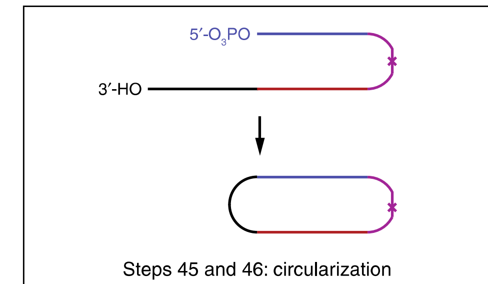


Juntawong et al., 2015

Reverse transcription

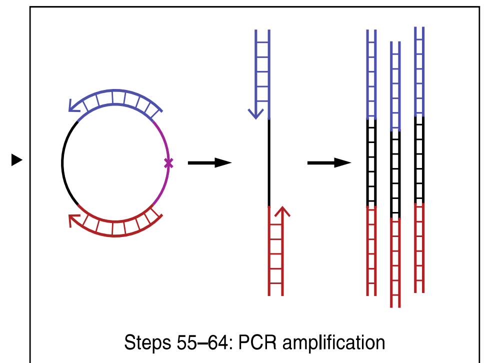


Circularization



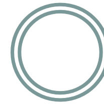
Steps 45 and 46: circularization

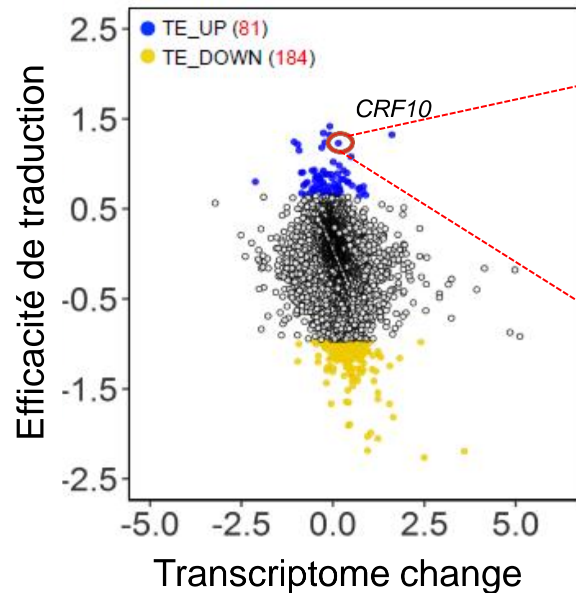
PCR

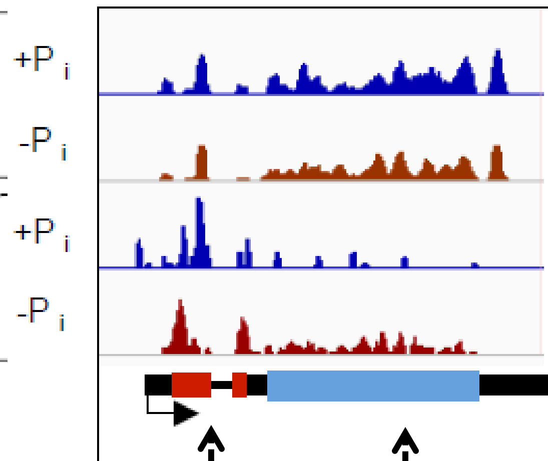


Steps 55–64: PCR amplification

RIBOSOME FOOPRINT SEQUENCING: RIBO-SEQ





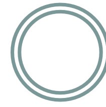


✓ Régulations traductionnelles des ARNm à l'échelle du génome

✓ Mecanismes de régulation: rôles des uORF

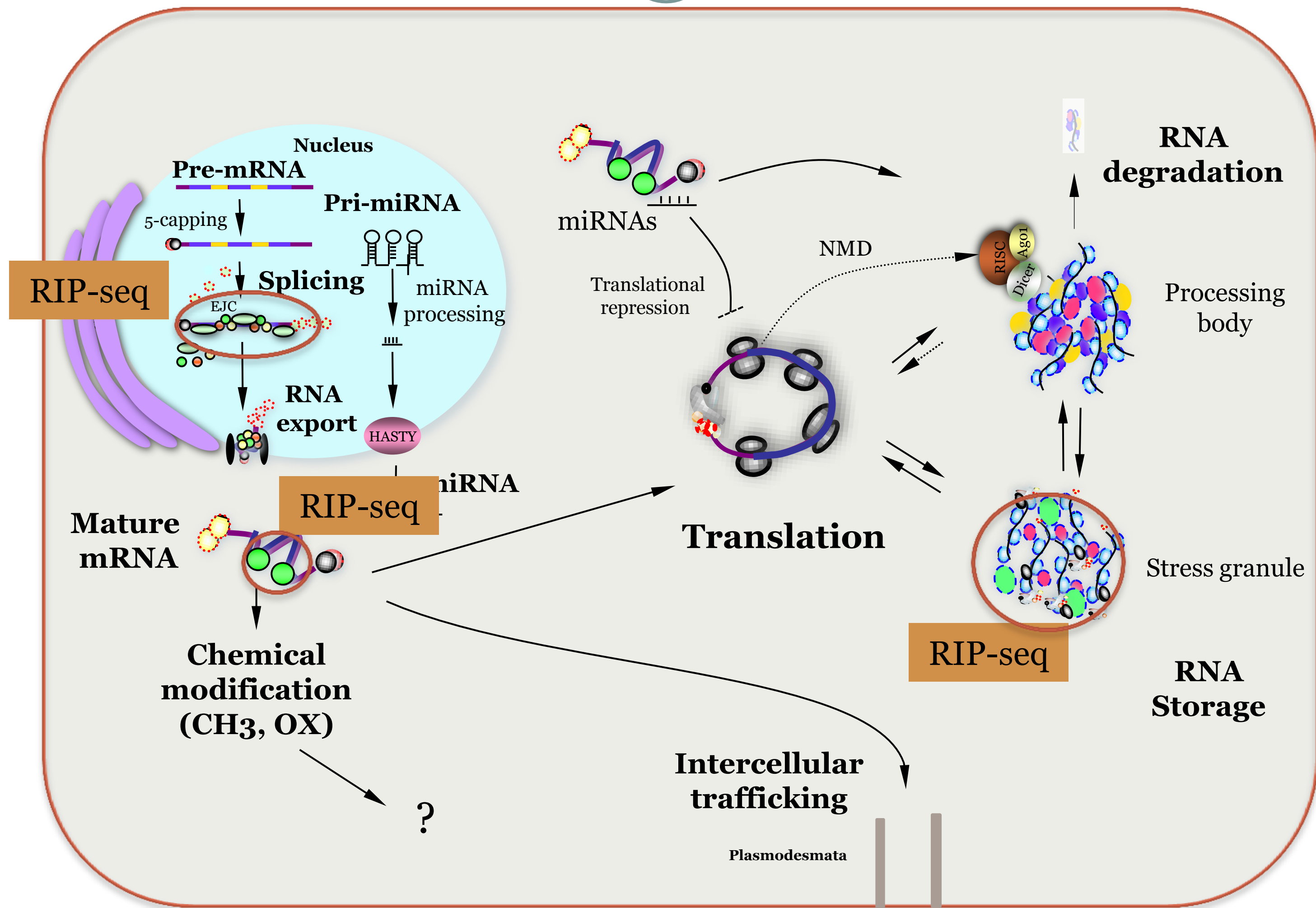
Bazin et al., under review

Ribosome fooprint sequencing: Ribo-seq

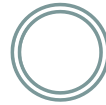


- A few recommendations:
  - Adjust RNAse1 digestion for each tissue type/Species
  - Use a standardized protocol, always the same reagents (specially RNAse1 batch)
  - Start with a relatively high amount of material (>= 10µg of RNAse1 digested RNA fragment)
  - Use ribo-zero kit to remove rRNA fragments from the ribosome footprints (can make up to 90% of the final read number!)

Q: Which RNA species are bound to an RNA binding protein ?

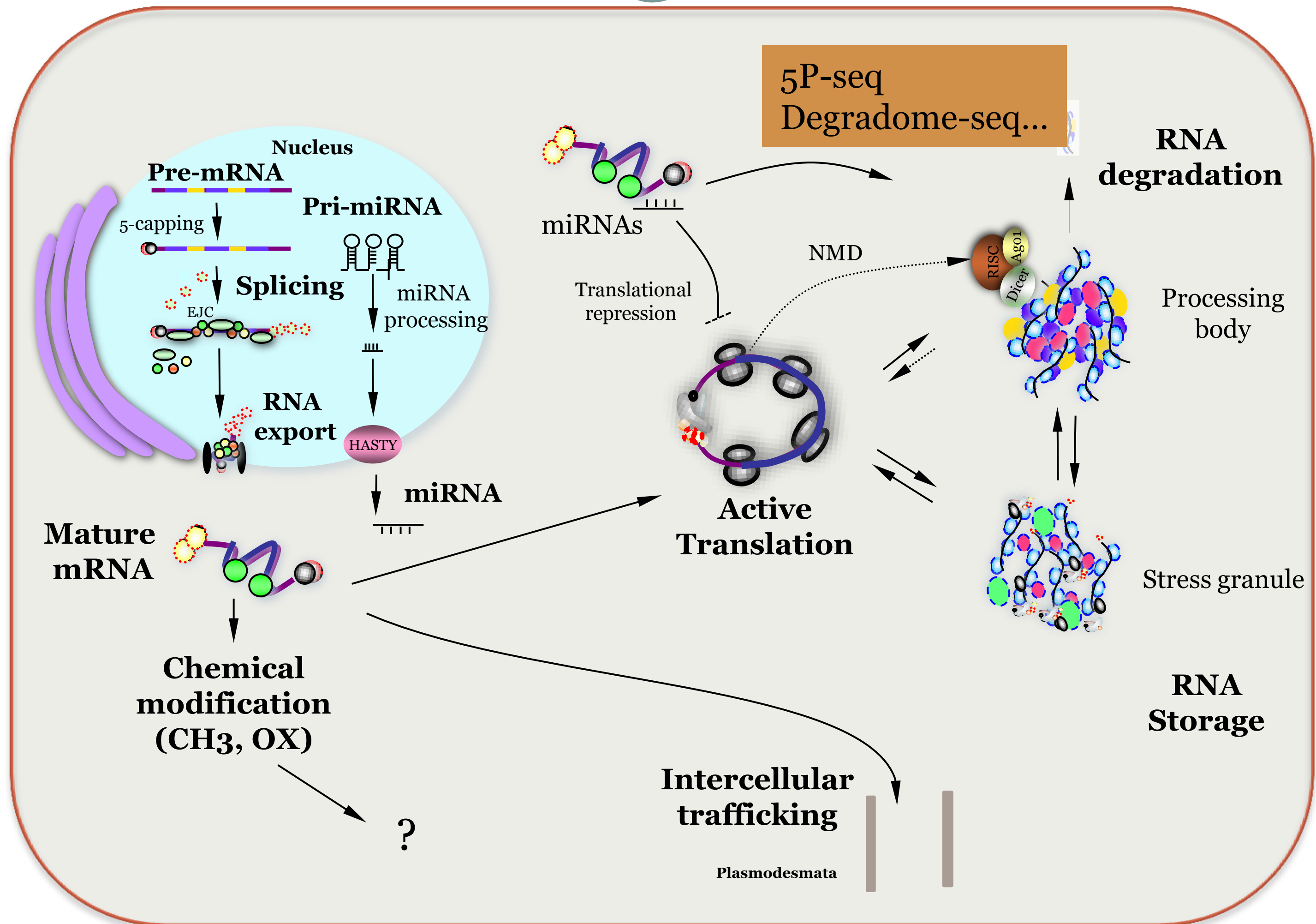


Q: Which RNA species are bound to an RNA binding protein?

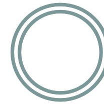


- Full length target RNA sequencing
  - RIP-seq : RNA immunoprecipitation
    - Analysis by RNA–seq of the RNA which co-immunoprecipitate with the target protein.
- RNA binding protein footprints
  - CLIP-seq : Cross-linking Immunoprecipitation :
    - Tissue cross-linking, immunoprecipitation and digestion of the unprotected RNA. Sequencing of the protected fragments is done using protocol similar to small RNA seq
  - Small RNA-seq reanalysis : Some small RNA sequencing contains footprint of RNA Binding Protein (eg PPR)

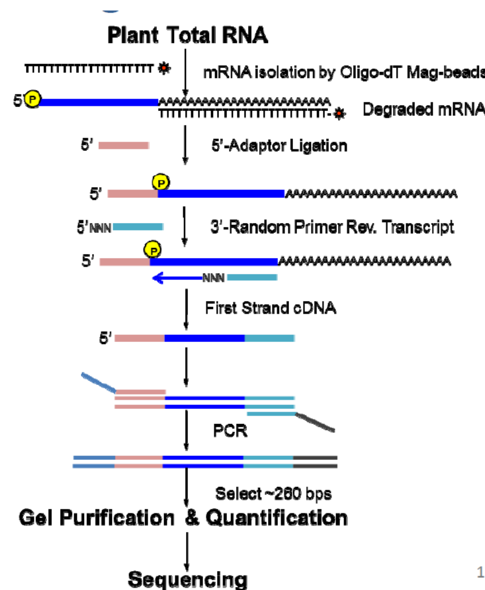
Q: Find the mRNA targets of microRNAs

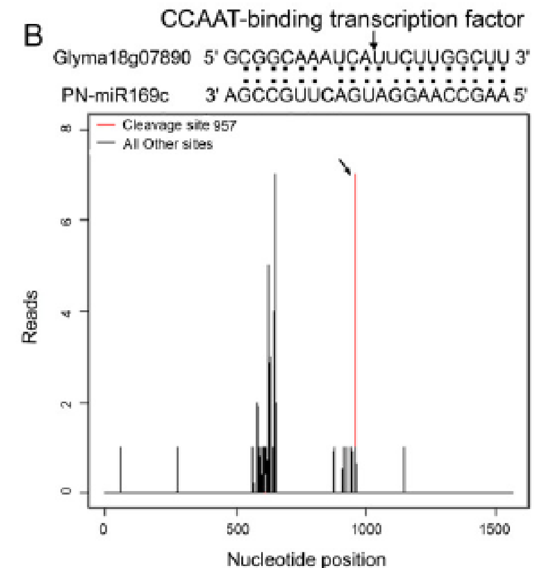


Q: Find the mRNA targets of microRNAs



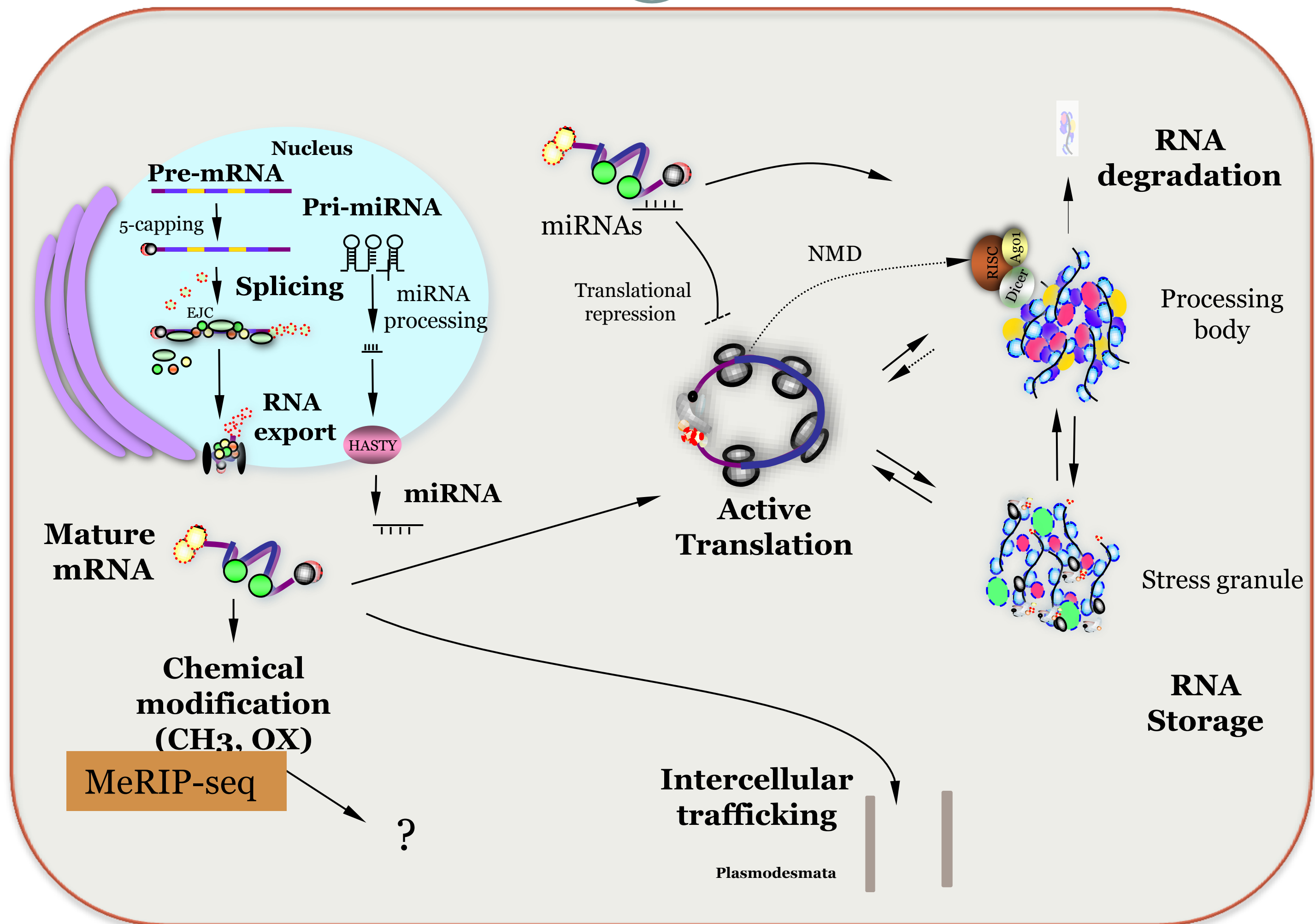
- Degradome sequencing/ 5P seq : Sequencing of mRNA decay intermediate
  - Based on chemical features of mRNA fragments :
    - PolyA tail / 5’P end



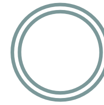


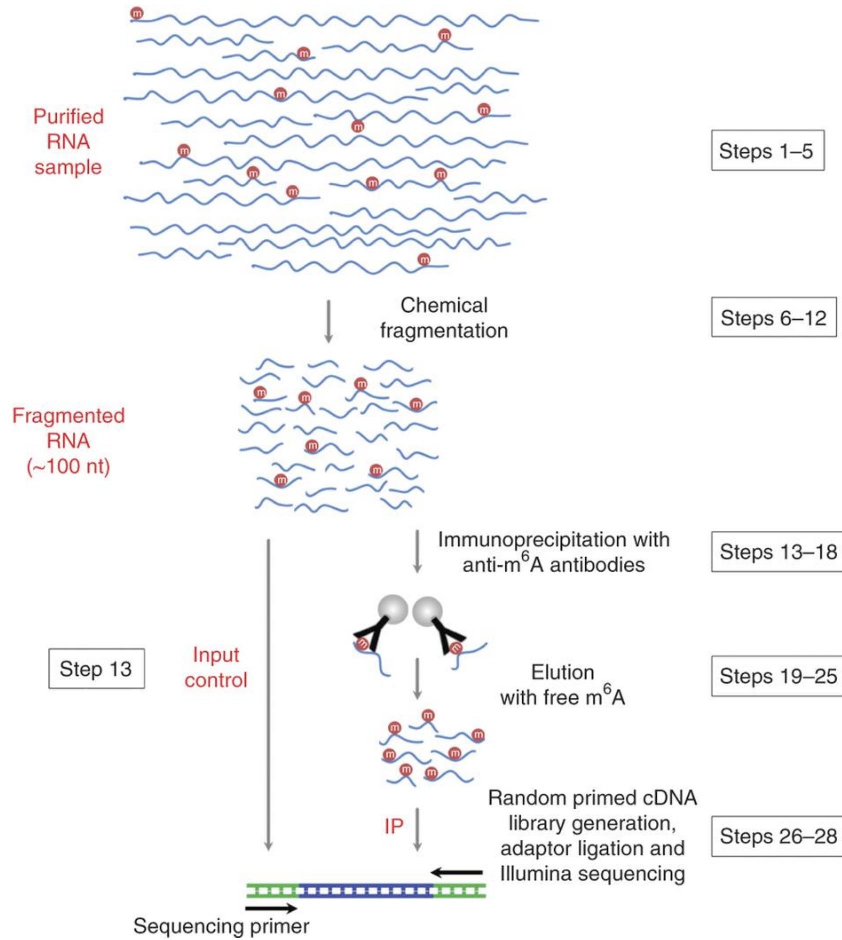
17

Identify/quantify methylated RNAs

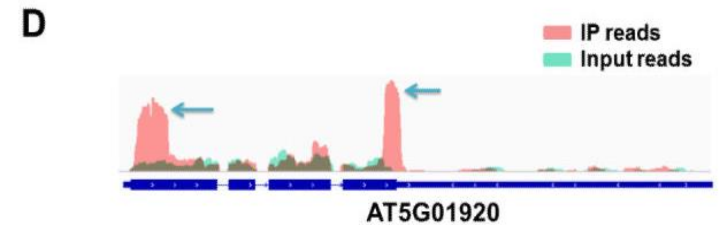


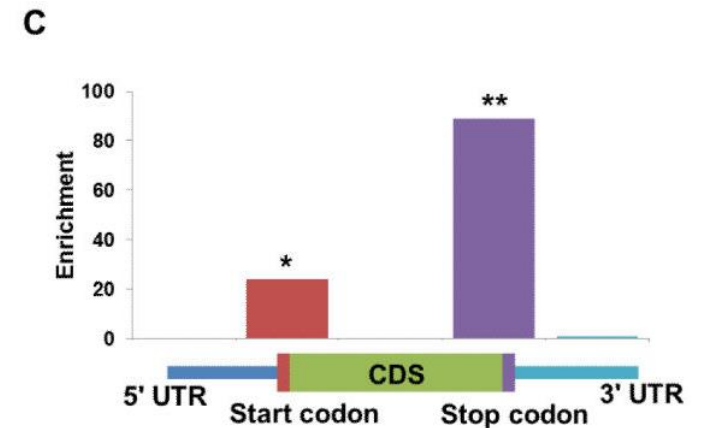
MeRIP-Seq





Dominissi et al., Nat. Prot 2013

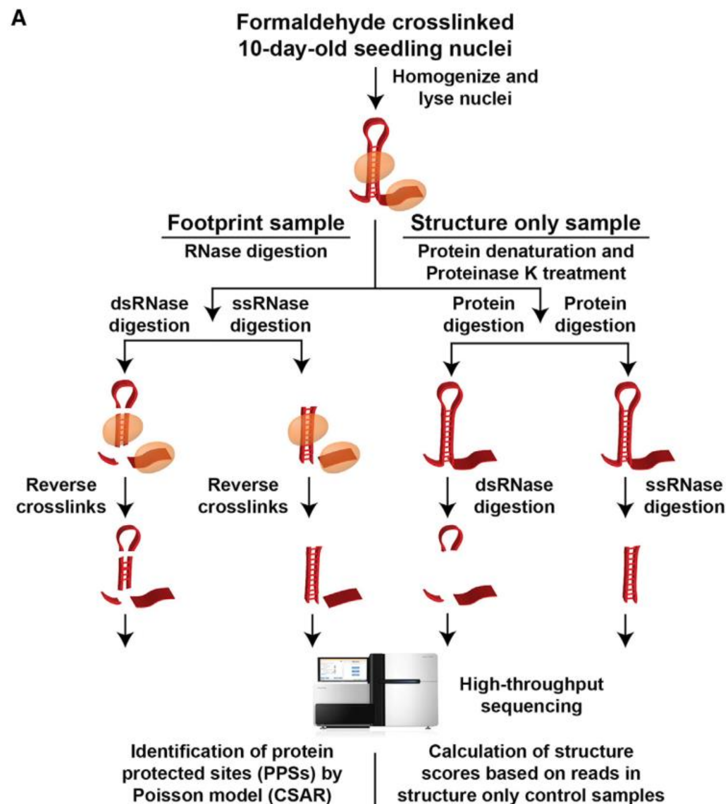




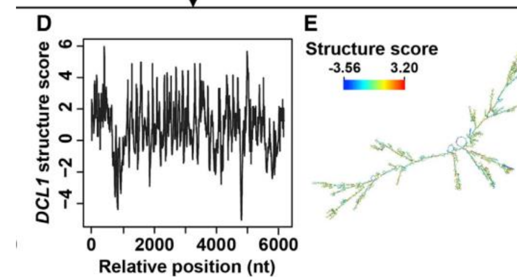
Luo et al., Nat. Com 2014

Probe RNA structure and protein binding sites ?

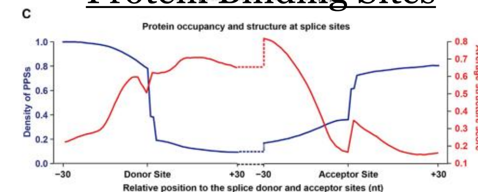
- Protein interaction profile sequencing : PIP-seq
  - Based on crosslinking and selective digestion of Protein / RNA complexes

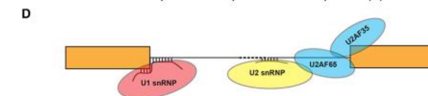


Structure Score / RNA folding



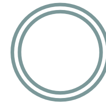
Protein Binding Sites



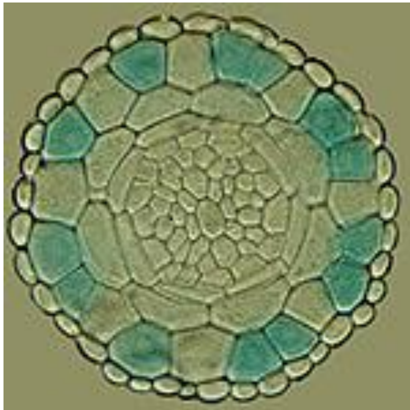


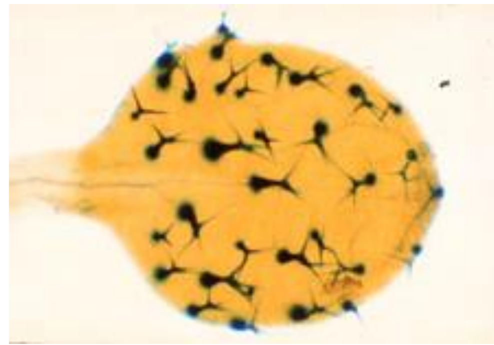
Anderson et al., 2016 (Current Prot. Plant. Biol)
Gosai et al., 2015 (Mol. Cell)

RNA POPULATIONS ARE DIFFERENTS IN DISTINCT CELL TYPE



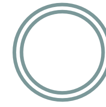
- Gene expression is highly variable in space



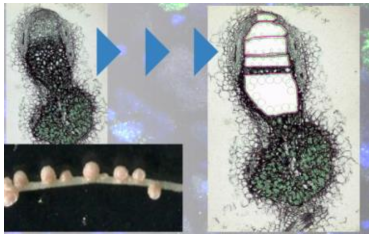


pGL2::GUS (Rerie et al., 1994)

High-Throughput Methods for Cell-Specific Gene Regulation Assessment



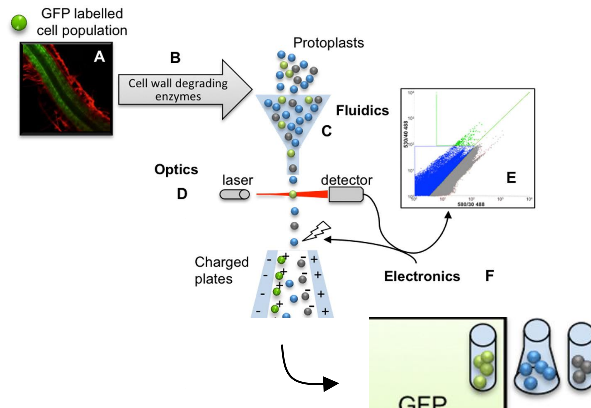
- Dissection-based and sorting-based methodologies :
  - Laser Capture Microdissection (LCM)



P.Gamas (LIPM, Toulouse)

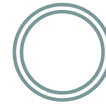
- Fixed tissue
- Time consuming
- Access to whole cell content

  - Fluorescence activated cell sorting (FACS)

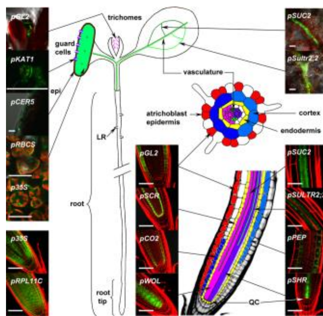


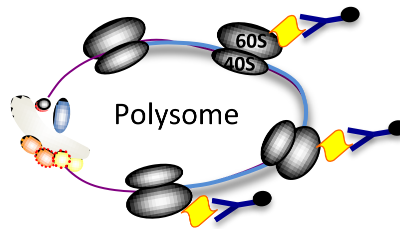
- Live tissue
- Give little RNA
- Stress during protoplast isolation
- Access to whole cell content

High-Throughput Methods for Cell-Specific Gene Regulation Assessment



- Affinity purification and immunoprecipitation :
  - Translating Ribosome Affinity Purification (TRAP)

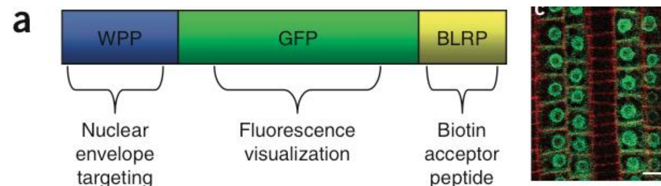




Mustroph et al. PNAS 2009

- Live tissue/*in vivo*
- Ribosome associated RNA
- Require transgenics

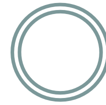
  - Isolation of Nuclei Tagged in Specific Cell Types (INTACT)



Deal and Henikoff., 2013

- Live tissue/*in vivo*
- Nuclear RNA/Chromatin
- Require transgenics

RNA-SEQ FROM LIMITED AMOUNT OF RNA

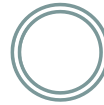


- Cell-specific Approach = limited amount of RNA
  - Require optimization of RNA protocols

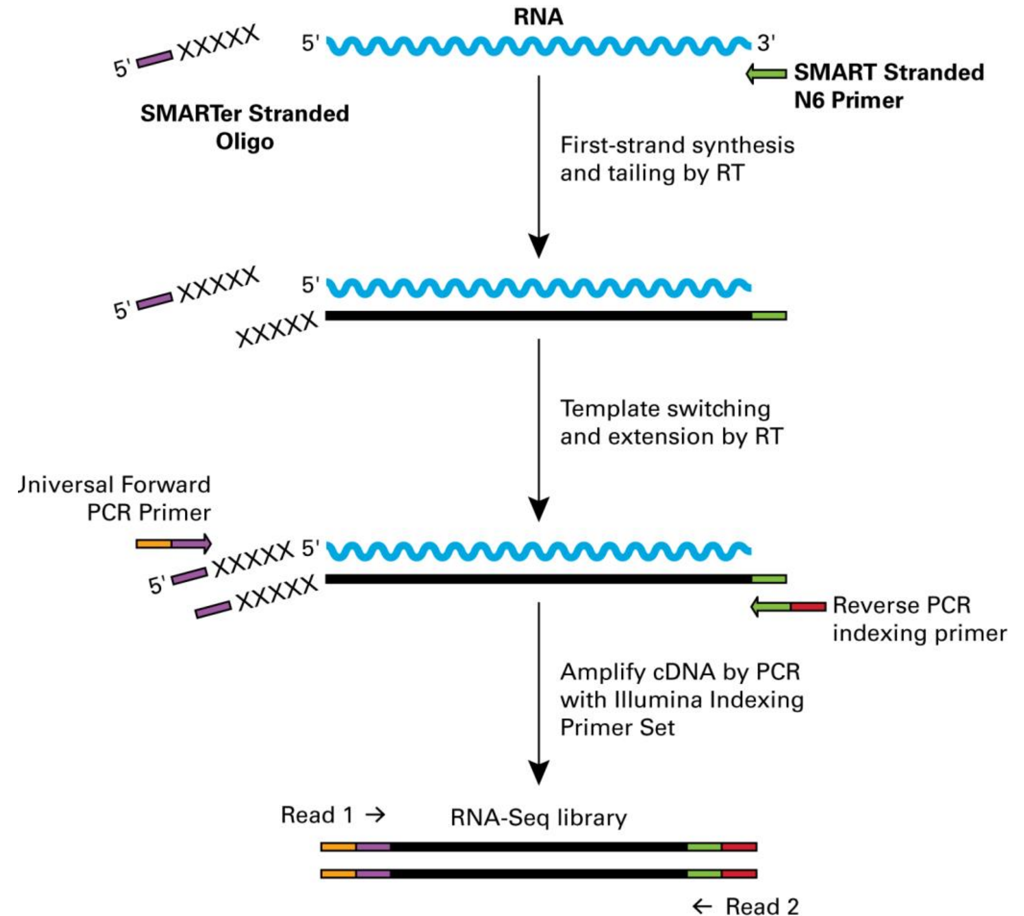
    - If total RNA quantity is >= 10 ng :

      - Protocols / Kits optimized for reduced RNA amount
        - Illumina Script-seq V2
        - Smarter Stranded (Clonetech)
        - NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina
        - Townsley et al., 2015 (Front. Plant Sciences)

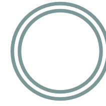
RNA-SEQ FROM LIMITED AMOUNT OF RNA



- Smarter Stranded (Clonetech)
  - Very simple design (Smart!)
  - Based on template switching activity of RT
  - Reduced purification steps



RNA-seq from limited amount of RNA

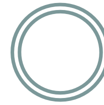


- Cell-specific Approach = limited amount of RNA
  - Require optimization of RNA protocols

    - If total RNA quantity is <= 1 ng :

      - Protocols / Kits including an additional RNA amplification step
        - Smarter Ultra Low Input RNA (Clonetech)
        - Smart-seq2 (Picelli et al., Nat. Prot. 2013)
        - Ovation RNA-seq System V2 (NuGEN)

RNA-SEQ FROM LIMITED AMOUNT OF RNA



- Smarter Ultra Low Input RNA (Clonetech)

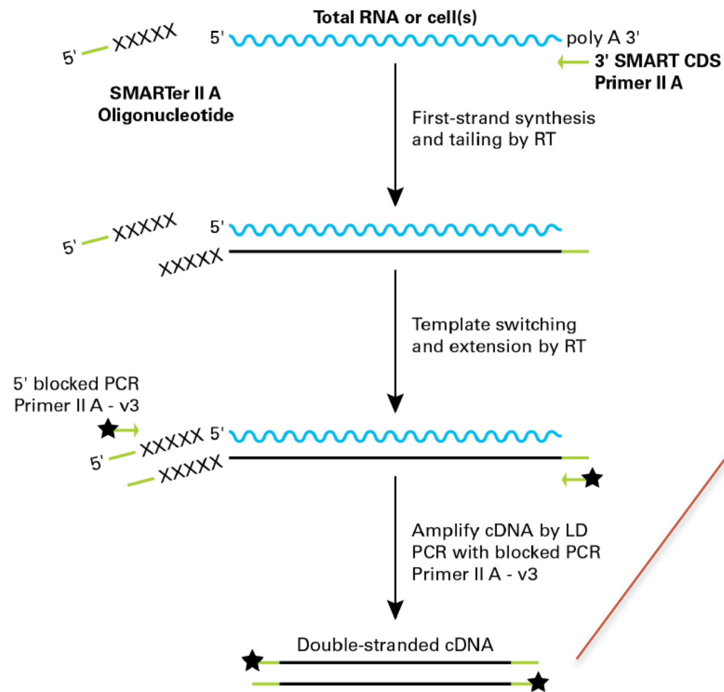
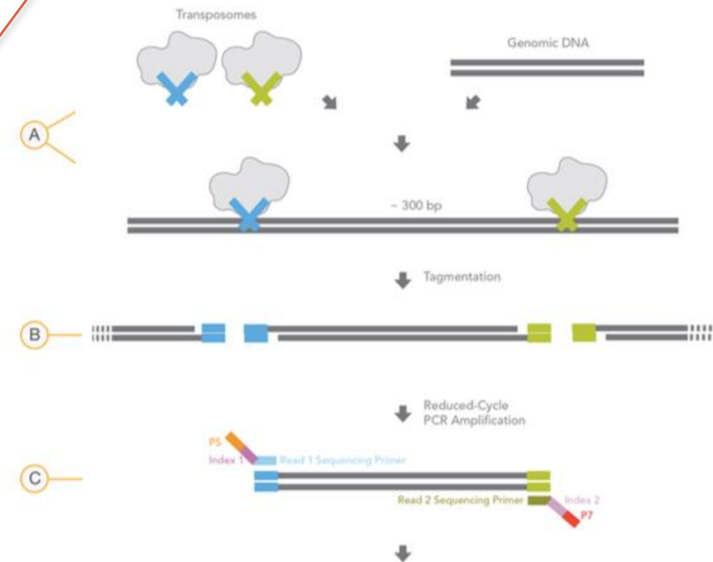
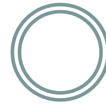


Figure 2. Flowchart of SMARTer cDNA synthesis. The SMARTer II A Oligonucleotide, 3' SMART CDS Primer II A, and PCR Primer II A - v3 all contain a stretch of identical sequence.

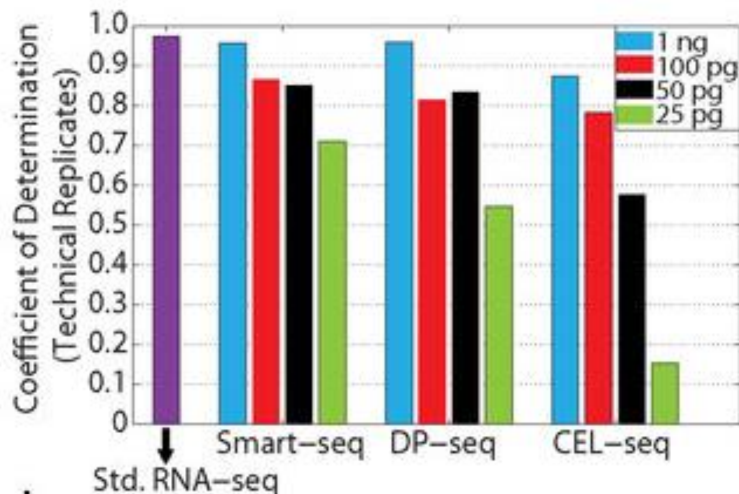
« DNA seq library »

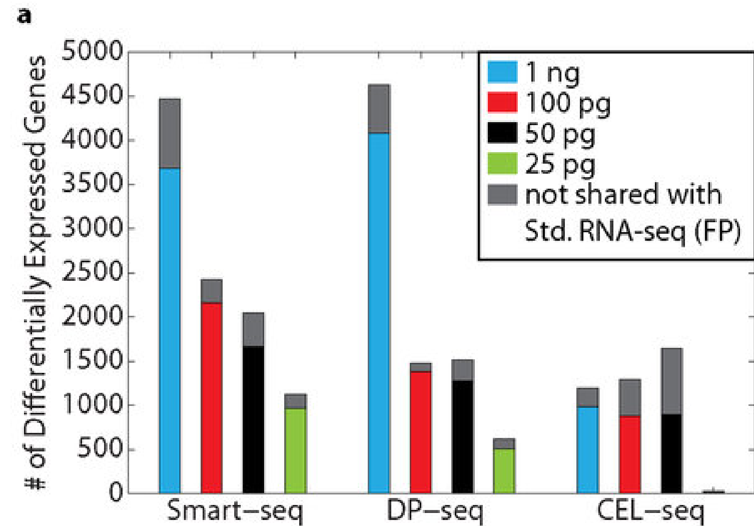


RNA-SEQ FROM LIMITED AMOUNT OF RNA



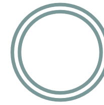
- RNA amplification from very low amount of RNA can introduce variabilty in the data

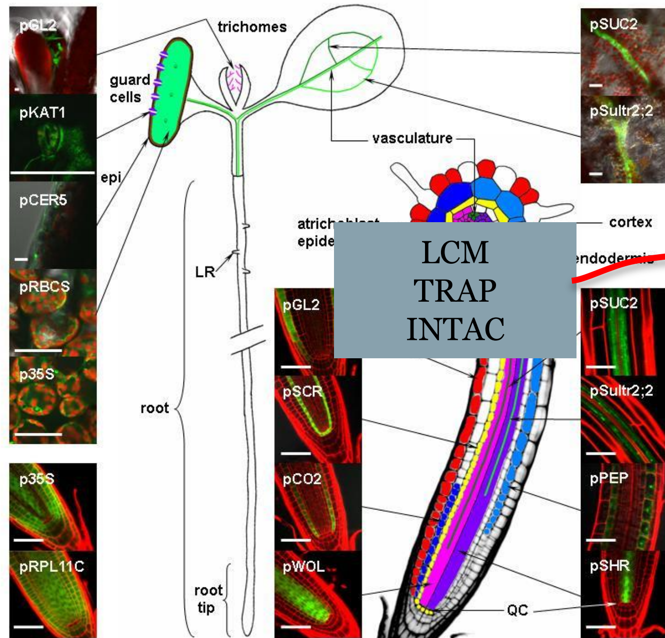


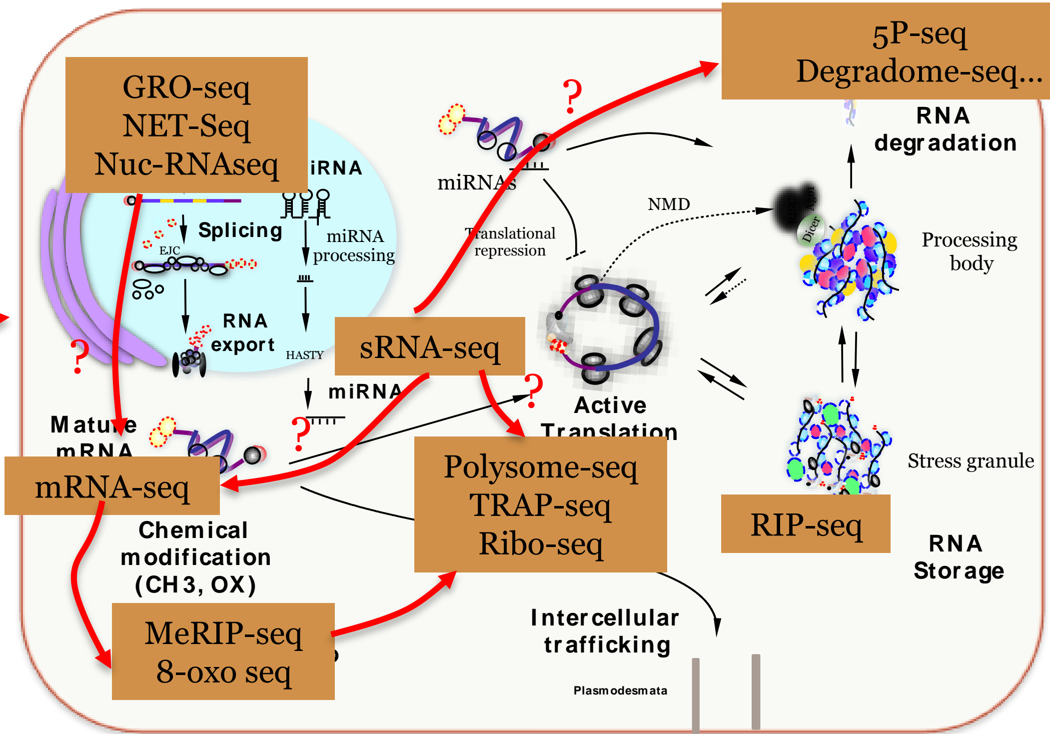


Barghava et al., 2014

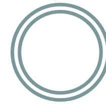
GENOME-SCALE, CELL-SPECIFIC MONITORING OF MULTIPLE GENE REGULATION TIERS USING RNA-SEQ







CONCLUSIONS



- An RNA-seq library is just cDNA fragments between adapters.
- There are always biases in genome-wide technologies including RNA-seq: Just keep the same bias for all samples
- One can sequence subpopulations of RNA based on their chemical feature, localization and protein associations
- By combining RNA-seq approaches and system biology, one can answer complex biological questions